

Moderate alcohol consumption, adiponectin, inflammation and type 2 diabetes risk

Prospective cohort studies and
randomized crossover trials

Michel M. Joosten

Thesis committee

Thesis supervisor

Prof. dr. R.F. Witkamp

Professor of Nutrition and Pharmacology, Wageningen University

Thesis co-supervisor

Dr. H.F.J. Hendriks

Senior scientist, TNO Quality of Life, Zeist

Other members

Dr. E.B. Rimm, Harvard School of Public Health, Boston, MA, U.S.A.

Prof. dr. ir. E.J.M. Feskens, Wageningen University

Prof. dr. ir. R.P. Mensink, Maastricht University, Maastricht

Prof. dr. ir. L.M. Havekes, Leiden University Medical Center, Leiden

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Michel M. Joosten

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Aan mijn ouders

If you admire someone, you should go ahead and tell them,
people hardly ever get the flowers while they can still smell them

Abstract

Background: Moderate alcohol consumption has been associated with a lower risk of type 2 diabetes in various populations. However, the underlying mechanisms are not entirely clear. The aims of this thesis were 1) to substantiate the evidence of the association between alcohol consumption and type 2 diabetes in observational research and 2) to examine physiological mechanisms in randomized trials with specific attention to adiponectin, inflammation and insulin sensitivity which may mediate the association between alcohol consumption and type 2 diabetes.

Methods: Two prospective cohort studies, one among 38,031 U.S. men (age: 45-75 y) of the Health Professionals Follow up Study (HPFS) and one among 35,625 Dutch men and women (age: 20-70 y) of the European Prospective Investigation into Cancer and Nutrition (EPIC)-NL cohort. Four randomized, crossover trials of eight to twelve weeks with consumption of 25-30 g alcohol/day in the form of vodka with orange juice, beer, or white wine (twice) compared to orange juice, alcohol free beer, white grape juice or water among 24 young men, 24 premenopausal and 36 and 22 postmenopausal women, respectively.

Results: A 7.5 g/day increase in alcohol consumption over four years was associated with lower diabetes risk among initial non-drinkers (hazard ratio [HR]: 0.78; 95% confidence interval (CI): 0.60, 1.00) and drinkers initially consuming <15 g/d (HR: 0.89; 95% CI: 0.83, 0.96) but not among men initially drinking ≥ 15 g/d (HR: 0.99; 95% CI: 0.95, 1.02) ($P_{\text{interaction}} < 0.01$) in U.S. men. Among Dutch subjects with ≥ 3 out of 4 low-risk lifestyle behaviors, moderate alcohol consumption was associated with a lower risk of type 2 diabetes compared with abstinence (HR: 0.56; 95% CI: 0.32, 1.00).

In the randomized crossover trials, alcohol consumption consistently increased circulating adiponectin levels by about 10% compared to abstinence ($P < 0.05$) regardless of beverage type, gender or age. These increases were evident after a minimum of three weeks of alcohol consumption. Moderate alcohol consumption also increased expression of the gene encoding adiponectin in adipose tissue and lowered serum fasting insulin and triglyceride levels (all $P < 0.05$). An integrated approach of large-scale profiling of proteins and genes revealed that moderate alcohol consumption for four weeks altered gene expression profiles of white blood cells and circulating markers related to inflammation in men (all $P < 0.05$). However, we did not observe an attenuated inflammatory response after a low-dose *in vivo* endotoxin bolus, despite increased high-density lipoprotein cholesterol and apolipoprotein levels after four weeks of alcohol consumption compared to abstinence. Six minutes of oral white wine exposure without swallowing substantially ($\sim 20\%$; $P < 0.05$) and temporarily (~ 20 min) decreased circulating free fatty acid concentrations compared with oral water exposure.

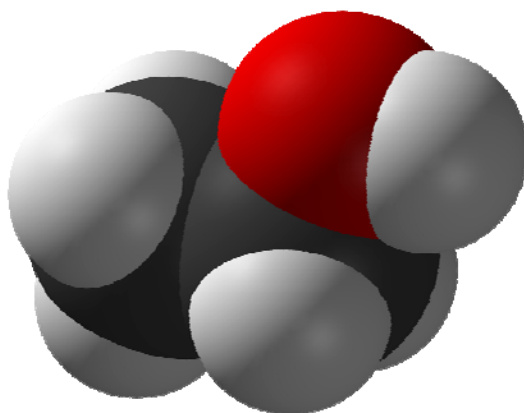
Conclusions: Moderate alcohol consumption was associated with a lower risk of type 2 diabetes compared with abstinence. The association persisted among subjects already at low risk based on combined favorable lifestyle behaviors. Also, increases in alcohol consumption among initially rare and light drinking men were associated with higher adiponectin levels and lower risk of type 2 diabetes. Increased adiponectin levels, anti-inflammatory effects and decreased insulin and triglyceride levels, may partially explain the inverse association between moderate alcohol consumption and type 2 diabetes.

Table of contents

1.	General introduction	11
Prospective cohort studies		
2.	Changes in alcohol consumption and subsequent risk of type 2 diabetes in men <i>(Diabetes; accepted)</i>	31
3.	Combined effect of alcohol consumption and lifestyle behaviors on risk of type 2 diabetes <i>(American Journal of Clinical Nutrition 2010; 91:1777-83)</i>	49
Randomized crossover trials		
4.	Moderate alcohol consumption increases insulin sensitivity and <i>ADIPOQ</i> expression in postmenopausal women: a randomized, crossover trial <i>(Diabetologia. 2008; 51:1375-81)</i>	69
5.	Moderate alcohol consumption alters leukocyte gene expression profiles and proteins related to immune response and lipid metabolism in men <i>(Submitted in revised form)</i>	85
6.	Alterations in total and high-molecular weight adiponectin after three weeks of moderate alcohol consumption in premenopausal women <i>(Submitted in revised form)</i>	103
7.	Alcohol-induced increase in lipoproteins does not alter inflammatory response after low-dose <i>in vivo</i> endotoxin challenge in men <i>(Submitted in revised form)</i>	115
8.	Short-term oral exposure to white wine transiently lowers serum free fatty acids <i>(Appetite. 2010; 55:124-9)</i>	131
9.	General discussion	147
	Summary	171
	Lay summary in Dutch (Samenvatting voor niet-ingewijden)	175
	Acknowledgement (Dankwoord)	181
	About the author	187

Chapter 1

General introduction



Alcohol consumption: what's (in) a drink?

The term 'alcohol' in alcohol consumption commonly refers to ethanol or ethyl alcohol, a 2-carbon alcohol, which is the dominant bioactive compound in all alcoholic beverages. It is safe to say that alcohol is both a tonic and a poison. The difference lies mostly in the dose. Moderate alcohol consumption is associated with lower total mortality (1), cardiovascular diseases (2;3) and type 2 diabetes (4) but also with increased breast cancer risk among women (5) compared with abstention. Excessive alcohol consumption is associated with an increased risk of injuries and accidents (6;7), liver diseases (8;9), several forms of cancer (5), alcohol dependence and alcoholism. The quantity of alcohol consumed thus determines how it affects overall health and well-being.

In 2006, the Dutch Health Council stated that "People who consume alcohol are well advised to limit their intake; adult men should drink no more than two standard Dutch units per day, and adult women no more than one unit. Binge drinking should be avoided. Alcohol consumption is inadvisable for the under-eighteens, and for pregnant women, women who are seeking to become pregnant and women who are breastfeeding" (10). According to the 2005 United States Department of Agriculture (USDA) Dietary Guidelines (11), moderate drinking is considered to be no more than one drink per day for women and no more than two drinks/day for men. Guidelines for sensible drinking set by the U.K. government in 1995 are 3 to 4 units or less for men and 2 to 3 units or less for women (12).

In the Netherlands a standard unit of beer (250 mL), wine (100 mL) and spirits (35 mL) all contain around 10 g of pure alcohol. In the United States typical serving sizes consist of one 12-ounce bottle of beer, one 5-ounce glass of wine, or 1.5 ounces of 80-proof distilled liquor equaling around 14 to 15 g of alcohol per serving (13) whereas a standard unit of alcohol in the United Kingdom equals 8 g of alcohol. Thus, at the upper limits, moderate drinking would be between 10 to 20 g of alcohol/day for women and 20 to 30 g/day for men, depending which guideline one uses.

Given differences in first-pass metabolism, volume of distribution, and overall body size between men and women (14), women experience toxic effects at approximately half the daily dose of alcohol of men. This notion is reflected in sex-specific limits that these above mentioned guidelines endorse.

Diabetes mellitus: etiology

Diabetes mellitus, often simply referred to as diabetes, is a disorder characterized by hyperglycemia (chronic elevated blood glucose levels) from defects in insulin secretion, insulin sensitivity, or both. These high blood glucose levels produce the classical symptoms of polyuria (frequent urination), and polydipsia (increased thirst). Diabetes is associated with increased morbidity and mortality. Complications include retinopathy, neuropathy and

renal failure. Several forms of diabetes can be distinguished. Type 1 diabetes mellitus, also known as insulin-dependent diabetes, is caused by defects in pancreatic function leading to an inability to produce sufficient amount of insulin. This type of diabetes is often diagnosed at early age and accounts for less than 5% of the diabetes cases.

Type 2 diabetes mellitus, formerly non-insulin-dependent diabetes mellitus or adult-onset diabetes, is the most common form of diabetes and accounts for approximately 90 to 95% of the cases of diabetes. As of 2000 at least 171 million people worldwide suffer from diabetes, or 2.8% of the population (15). Type 2 diabetes is a metabolic disorder that is characterized by high blood glucose in the context of insulin resistance and abnormal insulin secretion. It is caused by a combination of an impairment of the β -cells to release sufficient insulin (β -cell dysfunction) and an impaired metabolic response of peripheral tissues to circulating insulin (insulin resistance).

The underlying mechanisms leading to insulin resistance and β -cell dysfunction are still subject of research. Recently, obesity has been associated with a chronic low-grade inflammatory state of adipose tissue (16;17) in which circulating concentrations of inflammatory mediators such as tumor necrosis factor alpha (TNF- α) interleukin-6 (IL-6) and C-reactive protein (CRP) are increased (18). Low-grade inflammatory changes have been shown to precede type 2 diabetes by many years (19;20).

Besides elevated glucose concentrations, circulating free fatty acid (FFA) concentrations are also increased in obese persons, which lead to a reduction of glucose uptake in adipose tissue and skeletal muscles and in a stimulation of glucose output from the liver. In response to elevated glucose and FFA concentrations various tissues and cell types have been shown to release inflammatory mediators.

Finally, adipose tissue is known to secrete various signaling peptides – the adipokines – influencing insulin sensitivity, inflammation and food intake (21). The most abundant adipokine is adiponectin. Adiponectin is thought to be involved in glucose and lipid metabolism (22). Higher adiponectin levels have been consistently associated with a lower risk of type 2 diabetes (23) and cardiovascular disease (24). Moreover, adiponectin is positively associated with insulin sensitivity (25) and inversely with inflammatory markers (26).

Modifiable lifestyle factors

Although genetic elements are involved in the development of pre-diabetes and type 2 diabetes, the rapid changes in incidence rates during the last decades suggest a particularly important role for lifestyle related factors. Compelling evidence from epidemiologic studies and lifestyle interventions indicate that the current worldwide diabetes epidemic is largely due to changes in diet and lifestyle (27). The majority (~90%) of incidence type 2 diabetes cases may be prevented by a healthy lifestyle of which excess adiposity is by far the most important risk factor for diabetes (28-30). Other components of a low-risk lifestyle being physically active (31;32), refraining from smoking (33) and eating a diverse and healthy

diet rich in fruits, vegetables, whole grains, nuts and legumes and low in red and processed meats, sugar-sweetened beverages and sodium (34-37).

Besides these four modifiable lifestyle factors, moderate alcohol consumption could be regarded as a fifth low-risk factor to reduce the risk of type 2 diabetes.

State of the art evidence on alcohol consumption and type 2 diabetes risk

Substantial epidemiological evidence of at least 20 prospective studies (28;30;38-68) from different geographical regions and large and diverse cohorts described in several meta-analyses (4;69-73) links moderate alcohol consumption with a lower risk of type 2 diabetes. The population size and diversity and length of follow-up all vary greatly from study to study which adds to the robustness and generalizability of the findings. Most studies describe a J-shaped or U-shaped curve, whereby light to moderate drinkers have less risk than abstainers and heavy drinkers. The strength of the association described in these meta-analyses varies between the 10 and 40%. Maximum risk reduction is observed when consuming alcohol at about 25 g/day. No risk reduction was reported at consumption levels greater than 50 g/day for both men and women (4;69-73).

The ethanol itself, rather than specific components of various alcoholic beverages, appear to be the major factor in conferring health benefits. However, the alcohol-containing beverage which is most frequently consumed in specific populations, will be associated with the greatest benefit in that population; e.g. wine is the most common source of alcohol in Dutch postmenopausal women and wine is therefore of most benefit (57) whereas if spirits or beer are consumed the most these beverages appear to have the strongest associations (45;61).

Factors modifying the relation between alcohol consumption and type 2 diabetes

Besides several lifestyle factors that are associated with diabetes risk itself, other factors have the potency to modify the relation between alcohol consumption and diabetes risk. Two examples of such factors include drinking pattern and genetic predisposition.

Drinking pattern

Drinking pattern is an important factor in the relation, because the way alcohol is consumed has differential effects given the same volume: drinking moderate amounts per occasion and at regular frequency (4-7 days/week) decreases risk of type 2 diabetes (45;55) whereas binge drinking or episodic drinking (5 or more alcoholic beverages at a single occasion) increases the risk (46;59).

Genetic predisposition

Polymorphisms in genes that oxidize alcohol may also affect the relation between alcohol consumption and type 2 diabetes risk. The major enzymes involved are alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). Ethanol is oxidized to acetaldehyde in a reversible reaction catalyzed by the class I ADH isoenzymes (e.g. ADH1a, ADH1b, ADH1c) located in the cytosol of hepatocytes. Subsequently, acetaldehyde is then oxidized to acetate and water in a nonreversible reaction catalyzed by the mitochondrial class II ALDH2. Functional relevant polymorphisms are found in the genes encoding ADH1b and ADH1c, the latter affecting ethanol degradation rates and alcohol intake in Caucasian populations (74). Thus, such a polymorphism could modify the association of alcohol consumption with disease risk but with an effect anticipated only among drinkers. Indeed, it appears that the alcohol dehydrogenase 1c gene (*ADH1C*) modifies the relation between alcohol consumption and diabetes among drinkers (75) although some inconsistency exists (76).

Underlying mechanisms based on observational studies and randomized trials

Improved insulin sensitivity

There is substantial observational evidence that links alcohol consumption to improved insulin sensitivity either in a linearly inverse association (77-85) or a U-shaped relationship (47;76;86;87). This association even presents in subjects with confirmed type 2 diabetes (88). Furthermore, alcohol consumption is also inversely associated with glycemic control among type 2 diabetes patients (89) and complications of type 1 (90) and type 2 diabetes such as cardiovascular diseases and mortality (91).

Despite a large amount of observational studies showing a positive association between moderate alcohol consumption and insulin sensitivity, there is limited evidence from clinical trials that supports this finding. So far, eleven studies have examined the effects of moderate alcohol consumption on fasting glucose and insulin levels or (in)direct markers of insulin sensitivity in subjects without diabetes (92-102) (Table 1.1). Only one study reported lower steady-state plasma glucose (SSPG; a measure of insulin resistance) (102) in a subgroup of people after eight weeks of red wine or vodka consumption, equaling 30 g of alcohol/day. Another study reported lower fasting insulin and triglyceride levels, another independent risk factor for type 2 diabetes (94), after eight weeks of alcohol consumption in the form of 15 and 30 g pure alcohol and juice per day. The majority of studies, however, did not show changes in fasting levels of glucose and insulin or insulin sensitivity. It appears that the alcohol's effects on insulin sensitivity are only observed after prolonged periods of consumption (8 weeks) and are more pronounced in older, more insulin-resistant populations (94;102) or in subjects with type 2 diabetes (103-106).

Table 1.1: Clinical trials investigating changes in markers of insulin sensitivity after alcohol intake in subjects without type 2 diabetes.

Reference	Subjects (age)	Beverage type, (alcohol g/day)	Duration	Measure	Conclusion	Remarks
Cordain, 1997 (92)	14 men (21-50 y)	Red wine, (28 g/day)	6 weeks	Fasting insulin and glucose	No changes in fasting insulin and glucose.	- Only fasting measures. - Not specifically designed to measure IS
Cordain, 2000 (93)	20 overweight women (30-50 y)	Red wine, (20 g/day)	10 weeks	AIR, IVGTT, fasting insulin and glucose	Moderate wine consumption did not improve or impair IS	- 5 drinking days per week - Overweight subjects only
Davies, 2002 (94)	51 postmenopausal women (49-79 y)	Ethanol in juice, (0, 15 and 30 g/day)	8 weeks	Fasting insulin, glucose and triglyceride levels	Consumption of 15 and 30 g/day lowered insulin and triglyceride levels	Only fasting measures
Flanagan, 2002 (95)	21 men and women (21-41 y)	Vodka + mixer, (24 g/day)	1 week	AIR, IVGTT, fasting insulin and glucose	No difference in insulin sensitivity but difference in insulin secretion	Lower first phase insulin response after alcohol consumption
Zilkens, 2003 (96)	16 healthy men (20-65 y)	Beer, (~4.6 g/d) 12 x 375ml beer (32 g/week)	4 weeks	HOMA-IR, low dose infusion glucose insulin test	Reduction in alcohol intake from 7 to 0.8 drinks did not change IS	<i>Reducing</i> alcohol intake among heavy drinkers

Sierksma, 2004 (97)	23 middle aged men (45-65 y)	Whisky, (40 g/day)	17 days	Hyperinsulinemic euglycemic clamp	Moderate alcohol consumption improved IS in relatively insulin resistant subgroup	Correlation between adiponectin and IS in subgroup
Gonzalez-Ortiz, 2005(98)	8 young men (19-29 y)	Tequila, (9 g/day)	30 days	K _{ITT} , insulin, glucose, HOMA-IR	Detrimental metabolic changes after tequila.	- Nondrinkers, - 51% sugars (~24 g/day) in tequila
Beulens, 2006 (99)	34 men (35-70 y); 17 IS measurement	Red wine, (40 g/day)	4 weeks	Hyperinsulinemic euglycemic clamp	No effect on IS	No correlation adiponectin and insulin
Beulens, 2007 (100)	19 lean and overweight men (18-40 y)	Whisky, (40 g/day)	4 weeks	OGTT, Matsuda, fasting insulin and glucose	No effect on IS	Correlation between HMW adiponectin and skeletal muscle oxidative capacity
Beulens, 2008 (101)	Overweight and lean men (18-25 y)	Beer, (40 g/day)	3 weeks	OGTT Matsuda, Gutt, Cederholm	No effect on IS	Correlation between IS, ghrelin and adiponectin
Kim, 2009 (102)	11 men and 9 women (35-65 y)	Red wine or vodka, (30 or 20 g/day)	3 weeks	SSPG, fasted insulin and glucose	8 weeks of alcohol consumption had minimal impact on enhancing IS	Improvement of SSPG in men (~11%) and in and subjects consuming 30 g alcohol/day (~10%)

AIR, Acute Insulin Response; IS, insulin sensitivity; IVTT, Intravenous Glucose Tolerance Test; K_{ITT}, Constant of insulin tolerance test; OGTT, oral glucose tolerance test; SSPG, Steady state plasma glucose.

Increased circulating adiponectin levels

Second, alcohol consumption has been associated with higher circulating adiponectin concentrations (82;107-109) even in diabetic subjects (88). Adiponectin was demonstrated to be an independent risk factor of insulin sensitivity and type 2 diabetes based on 10 prospective cohort studies (23). It is also inversely associated with markers of inflammation (26). More importantly, increased adiponectin concentrations may explain up to one third of the association between alcohol consumption and type 2 diabetes (110).

Also in several randomized trials, predominantly performed in males, moderate alcohol consumption increased plasma total adiponectin concentrations (97;99-101;111) and even the high-molecular weight (HMW) adiponectin isomer (100). Especially the latter of adiponectin has been proposed as the most active metabolic form (112-114).

Anti-inflammatory effects

Third, moderate alcohol consumption has various effects on the immune system (115) and is associated with lower levels of inflammatory markers (116-118). In concord with insulin sensitivity and adiponectin levels, the association between alcohol consumption and inflammatory markers even persists among men with type 2 diabetes (88;119).

Data from randomized trials on inflammatory markers after prolonged moderate alcohol consumption are scarce but do suggest lower levels of CRP (120;121) and certain cytokines (122) although not consistently (123). Furthermore, moderate alcohol consumption also has several acute effects on white blood cells. Also several lines of evidence suggest that acute moderate alcohol consumption inhibits the pluripotent transcription factor nuclear receptor regulatory factor kappa B (NF- κ B) in white blood cells (124;125). Activation of this transcription factor is a pivotal step in the induction of inflammatory cytokines, chemokines and growth factors.

Beneficial postprandial effects

Recent evidence showed that alcoholic beverages as such are capable of reducing peak blood glucose concentrations and the overall postprandial glucose response when consumed with or before a carbohydrate-containing meal (126;127). Besides lowered glycemic responses, acute alcohol consumption also decreased serum FFA levels (128) also in subjects with type 2 diabetes (129).

Rationale and outline of this thesis

There is ample evidence from observational research linking moderate alcohol consumption to a reduced risk of type 2 diabetes. However, several aspects have not yet been studied. Also, the physiological mechanisms that account for this observation are not completely understood. We thus aimed to:

1) further substantiate the existing literature of prospective cohort studies on alcohol consumption and risk of type 2 diabetes by investigating the temporality of the relation between alcohol intake and type 2 diabetes risk and by examining this association among subjects already at low risk of diabetes due to individual and joint contribution of healthy lifestyle behaviors

2) examine physiological mechanisms in short-term intervention studies of alcohol consumption that potentially mediate the association between alcohol consumption and type 2 diabetes. Of particular interest is the effect of alcohol consumption on adiponectin concentrations, low-grade inflammatory processes and to a lesser extent insulin sensitivity.

Prospective cohort studies

A new approach to explore the causality of the alcohol–diabetes relation is to examine the temporality of the association; in other words: does the introduction or removal of alcoholic beverages lead to a change in risk? In **chapter 2** we examine the effect of changes in alcohol consumption on subsequent changes in risk of type 2 diabetes during twenty years of follow-up. Furthermore, we look at associations between a four-year change in alcohol consumption and its effect on circulating levels of adiponectin and hemoglobin A_{1c} (HbA_{1c}).

As previously described in this chapter approximately 90% of the incident type 2 diabetes cases could have been prevented by adhering to several low-risk lifestyle behaviors. It is uncertain whether alcohol consumption could still decrease the risk of diabetes even if people already pursue several of these healthy lifestyle behaviors and may consequently be considered as an independent risk factor. Therefore, we evaluate in **chapter 3** the association between alcohol consumption and type 2 diabetes in the context of single and multiple favorable lifestyle behaviors.

Randomized crossover trials

Despite the consistent decreased risk of type 2 diabetes associated with moderate alcohol consumption in observational studies the majority of the short-term alcohol-feeding trials do not find changes in insulin sensitivity, an important predictor of type 2 diabetes, even after prolonged moderate alcohol consumption. In **chapter 4** we try to confirm a previously observed finding of improved insulin sensitivity in postmenopausal women. Additionally, we investigate the effects of white wine on the expression of the gene encoding adiponectin (*ADIPOQ*) and on corresponding circulating protein levels of adiponectin, an important biochemical predictor of type 2 diabetes.

Moderate alcohol consumption has several acute effects on gene expression of white blood cells but longer term effects on gene expression are largely unknown. In **chapter 5** we thus

explore the effects of four weeks of moderate vodka consumption on changes in associated gene expression of leukocytes (white blood cells) and several circulating proteins.

Although prolonged moderate alcohol consumption increases circulating adiponectin levels acute alcohol consumption in moderation does not increase postprandial adiponectin levels. This suggests that longer periods of consumption are needed to increase adiponectin levels. Therefore, we describe in **chapter 6** weekly changes in two forms of plasma adiponectin levels during 3 weeks of moderate beer consumption in normal-weight premenopausal women.

Since a low-grade inflammation is thought to play an important role in the development of type 2 diabetes, we tried to mimic an acute low-grade inflammatory status by challenging young men with a low-dose endotoxin bolus to see whether the inflammatory response is attenuated after moderate vodka consumption. These results are described in **chapter 7**.

Besides prolonged and postprandial effects of moderate alcohol consumption, pre-prandial effects of alcohol consumption (e.g. mere tasting of alcohol before ingestion) might also affect glucose and lipid metabolism. These pre-prandial effects belong to a so called cephalic phase and are important in triggering gastric and intestinal responses. Therefore, in **chapter 8** we investigate the effects of pre-prandial oral white wine exposure on several markers of glucose and lipid metabolism to investigate whether alcohol induces cephalic phase responses.

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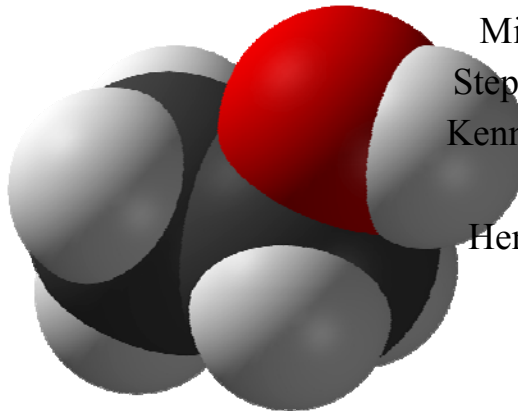
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Chapter 2

Changes in alcohol consumption and subsequent risk of type 2 diabetes in men



Michel M. Joosten
Stephanie E. Chiuve
Kenneth J. Mukamal
Frank B. Hu
Henk F.J. Hendriks
Eric B. Rimm

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Background: Alcohol consumption has been associated with lower risk of diabetes, but whether changes in alcohol consumption over time influence future diabetes risk is unclear.

Objective: The objective of this study was to investigate the association of four-year changes in alcohol consumption with subsequent risk of type 2 diabetes.

Design: We prospectively examined 38,031 middle-aged men from the Health Professionals Follow-up Study free of diagnosed diabetes or cancer in 1990. Alcohol consumption was reported on food frequency questionnaires and updated every four years.

Results: A total of 1,905 cases of type 2 diabetes occurred during 428,497 person-years of follow-up. A 7.5 g/day (~half a glass) increase in alcohol consumption over four years was associated with lower diabetes risk among initial nondrinkers (multivariable hazard ratio [HR]: 0.78; 95% confidence interval [CI]: 0.60, 1.00) and drinkers initially consuming <15 g/day (HR: 0.89; 95% CI: 0.83, 0.96) but not among men initially drinking ≥ 15 g/day (HR: 0.99; 95% CI: 0.95, 1.02; $P_{\text{interaction}} < 0.01$). A similar pattern was observed for levels of total adiponectin and hemoglobin A_{1c}, with a better metabolic profile among abstainers and light drinkers who modestly increased their alcohol intake, compared with men who either drank less or among men who were already moderate drinkers and increased their intake. Likewise, compared to stable light drinkers (0-4.9 g/day), light drinkers who increased their intake to moderate levels (5.0-29.9 g/day) had a significantly lower risk of type 2 diabetes (HR: 0.75; 95% CI: 0.62, 0.90).

Conclusions: Increases in alcohol consumption over time were associated with lower risk of type 2 diabetes among initially rare and light drinkers. This lower risk was evident within a four-year period following increased alcohol intake.

Introduction

Alcohol consumption has been consistently associated with a reduced risk of type 2 diabetes compared with abstinence or excessive consumption (1;2). Most prospective studies measure alcohol consumption at only one point in time which assumes intake is fairly stable over time. However, alcohol consumption is dynamic, especially over longer periods of follow-up (3). Importantly, changes in alcohol consumption over time have been associated with subsequent changes in risk of cardiovascular diseases (4-6) and mortality (7) although some inconsistency exists (8;9). Variability in intake over time thus highlights the constraints of single measures of alcohol consumption.

To our knowledge, no observational studies have examined the association between changes in alcohol consumption over time and future risk of type 2 diabetes, despite the importance of such studies both in assessing the robustness of the alcohol-diabetes association and in addressing a topic of direct clinical importance – what happens when individuals adopt or cease drinking? Short-term randomized trials of alcohol have shown changes in insulin sensitivity and adiponectin concentrations within six to eight weeks, suggesting changes in subsequent risk of diabetes could plausibly occur with a short latency (10-12). Therefore, we attempted to examine whether initiation of light to moderate alcohol consumption is associated with a lower subsequent risk of type 2 diabetes, and likewise whether reduction in alcohol consumption is associated with higher type 2 diabetes risk.

To accomplish these aims, we examined men enrolled in the Health Professionals Follow-up Study (HPFS), an ongoing prospective cohort of men who have repeatedly reported their alcohol consumption over time and in whom validated diagnoses of diabetes have been ascertained for two decades.

Methods

Study population

The HPFS is a prospective investigation of 51,529 U.S. male health professionals aged 40 - 75 y at baseline in 1986 who returned a mailed questionnaire about diet and medical history. Participants subsequently provided diet, lifestyle, and medical information on biennial questionnaires. We excluded men with missing data on body mass index (BMI) and physical activity at baseline. We also excluded men who had implausible nutritional information (≥ 70 missing food items or estimated daily energy intake < 800 or > 4200 kcal). Furthermore, we excluded men who deceased or had diagnosed diabetes or cancer (except non melanoma skin cancer) before follow-up for these analyses started (i.e., in 1990). After these exclusions 38,031 participants remained for the analyses.

To assess the association between changes in alcohol intake and biochemical markers of glycemia, we examined a subset of men in this cohort who participated in a nested case-control study of coronary heart disease. In 1994, 18,825 participants provided blood samples. Men who provided samples were somewhat younger but were otherwise similar to those who did not provide samples. We matched 266 men with incident coronary

heart disease until 2000 and an additional 188 cases from 2000 to 2004 with controls matched for age, date of blood draw, and smoking status on a 1:2 basis, as described previously (13;14). All participants gave written informed consent, and the Harvard school of public health human subjects committee review board approved the study protocol.

Assessment of alcohol consumption

In 1986, men reported their alcohol consumption on a semi quantitative food frequency questionnaire (FFQ) (15;16) that included 'separate items for beer, white wine, red wine, and liquor. Participants were asked how often, on average over the past year, they consumed each beverage. We calculated total alcohol intake by multiplying the average consumption of each beverage by the published alcohol content of the specified portion size based on periodically updated U.S. Department of Agriculture food composition tables and then summing across beverages (17). The FFQ was administered again every 4 years, with an item for light beer added in 1994. We assessed the validity of self-reported alcohol consumption by comparing estimated alcohol intake from the FFQ with the intake derived from two 7-day dietary records among 127 participants who returned questionnaires in 1986 and 1987 and resided in or near Boston, Massachusetts. The Spearman rank correlation coefficient between alcohol intake estimated from the FFQ and corresponding intake from diet records was 0.86 (18).

Assessment of lifestyle factors

Lifestyle factors were assessed biennially using questionnaires including BMI, smoking, physical activity, and medical conditions. Participants reported physical activity as the average time engaged in specific activities during the previous year. Reported weights have been shown to correlate well with measured weights, (16) and the assessment of physical activity was previously validated (19). We obtained energy intake, coffee consumption and energy-adjusted intakes of dietary fiber, glycemic load, *trans* fat, and the ratio between polyunsaturated and saturated fat from a semi quantitative FFQ (20). Glycemic load was calculated by multiplying the amount of carbohydrates by the average glycemic index as previously described (21).

Ascertainment of type 2 diabetes

Incident cases of type 2 diabetes were identified by self report and confirmed by a validated supplementary questionnaire detailing symptoms, diagnostic laboratory test results and diabetes treatment. The diagnosis was confirmed if participants reported at least one of the following: treatment with insulin or oral hypoglycemic medication, at least one classic symptom (excessive thirst, polyuria, weight loss, or hunger) plus elevated plasma glucose level, or at least two elevated plasma glucose concentrations on two different occasions in the absence of symptoms. Elevated plasma glucose concentration was defined as at least ≥ 140 mg/dL (≥ 7.8 mmol/L), plasma glucose ≥ 200 mg/dL [≥ 11.1 mmol/L] nonfasting, or plasma glucose ≥ 200 mg/dL [≥ 11.1 mmol/L] after ≥ 2 hours during an oral glucose

tolerance test before 1998; for cases diagnosed in 1998 and later, the fasting plasma glucose threshold was lowered to ≥ 126 mg/dL (≥ 7.0 mmol/L) (22). The validity of self reported diabetes has been confirmed with medical record review in a sample (23).

Measurement of biochemical variables

Blood samples were collected in liquid EDTA tubes, placed on ice packs, stored in Styrofoam containers, returned to our laboratory via overnight courier, and centrifuged and aliquoted for storage in liquid nitrogen freezers (-130°C or colder). Plasma total adiponectin concentrations were measured by competitive radio-immunoassay (Linco Research Inc, St Charles, MO, U.S.A.) for cases and controls ascertained through 2000 ($N = 798$). Hemoglobin A_{1c} (HbA_{1c}) concentrations were measured by turbidimetric immunoinhibition for cases and controls through 2004 ($N = 1,365$).

Statistical analysis

Each individual contributed person-time from the return of the 1990 questionnaire to the date of diagnosis of type 2 diabetes, date of diagnosis of cancer or death, or January 31, 2006, whichever came first. We used Cox proportional hazards models to calculate hazard ratios (HR) and 95% confidence intervals (CI). We used change in alcohol consumption updated every 4 years as a time-varying covariable, using an Anderson-Gill data structure (24). Thus, we used the change in alcohol consumption between the 1986 and 1990 questionnaires to determine the risk for type 2 diabetes during the period from 1990 to 1994, the change in alcohol consumption based on the 1990 and 1994 questionnaires for the period from 1994 to 1998, and so on. In these analyses, men contributed person-time only during each four-year period in which they provided data on alcohol consumption. We skipped contributions of person-time for individuals with missing information on alcohol consumption during follow-up for that specific period. In multivariate models, we adjusted for age (five categories), BMI (eight categories: <23.0 , $23.0-23.9$, $24.0-24.9$, $25.0-25.9$, $26.0-26.9$, $27.0-27.9$, $28.0-28.9$, $29.0-29.9$, $30.0-30.9$, $31.0-31.9$, $32.0-32.9$, $33.0-33.9$, $34.0-34.9$, or ≥ 35.0 kg/m²), physical activity (quintiles), smoking status (never, former, current 1-14 cigarettes/day, current 15-24 cigarettes/day or current ≥ 25 cigarettes/day), family history of type 2 diabetes (yes or no), incident and prevalent cardiovascular disease (stroke, myocardial infarction, coronary artery bypass surgery or angina), hypertension, and hypercholesterolemia, dietary glycemic load, fiber intake, *trans* fat intake, ratio of polyunsaturated to saturated fat (all four in quintiles and energy-adjusted), coffee intake (quintiles) and total energy intake (continuous). All variables were treated as time-varying covariates in our models. Linear trends across (change in) alcohol consumption categories were tested by treating the median value of each category as a continuous variable.

Table 2.1 : Characteristics according to 4-year change in alcohol consumption in 1990 stratified by initial intake in 1986 (N = 33,073)¹.

Initial alcohol consumption (g/day)	Change in alcohol consumption 4 years later (g/day)				
	Moderate to large decrease (≥7.5)	Small to moderate decrease (2.5–7.49)	No change / relatively stable (±2.49)	Small to moderate increase (2.5–7.49)	Moderate to large increase (≥7.5)
No drinks/d (0)					
No. (% of total) of participants	6,868 (20.8%)	309 (0.9%)	170 (0.5%)
Age (y)	57.8	56.7	59.4
BMI (kg/m ²)	25.6	25.5	25.7
Weight gain from '86 to '90 (lbs)	1.2	1.6	3.0
Alcohol consumption in 1986 (g/day)	0.0	0.0	0.0
Alcohol consumption in 1990 (g/day)	0.2	4.1	21.5
Physical activity (METs/week)	35.8	36.1	38.0
Current smoker (%)	3.4	3.7	5.0
Family history of diabetes (%) ²	15.9	14.3	11.2
Cardiovascular disease (%) ³	11.4	13.9	12.9
Hypertension (%)	25.6	26.3	25.3
Hypercholesterolemia (%)	28.4	35.9	29.2
<1 drink/day (0.1–14.9)					
No. (% of total) of participants	791 (2.4%)	3,517 (10.6%)	9,321 (28.2%)	2,391 (7.2%)	1,190 (3.6%)
Age (y)	58.9	57.3	57.1	56.7	57.6
BMI (kg/m ²)	25.5	25.5	25.5	25.4	25.7
Weight gain from '86 to '90 (lbs)	-0.2	1.0	1.3	1.6	1.7
Alcohol consumption in 1986 (g/day)	12.1	8.3	4.9	6.7	8.0

Alcohol consumption in 1990 (g/day)	2.3	3.9	4.7	11.2	24.5
Physical activity (METs/week)	37.8	38.6	36.1	40.3	38.4
Current smoker (%)	5.6	3.5	4.0	4.4	5.4
Family history of diabetes (%) ²	14.9	15.6	16.1	15.3	14.5
Cardiovascular disease (%) ³	10.9	11.6	11.3	10.9	10.1
Hypertension (%)	27.0	24.0	23.6	24.2	27.0
Hypercholesterolemia (%)	32.3	30.9	30.7	31.2	33.2

≥1 drink/day (0.1-14.9)					
No. (% of total) of participants	3,256 (9.8%)	1,498 (4.5%)	2,118 (6.4%)	665 (2.0%)	979 (3.0%)
Age (y)	58.7	57.7	58.8	57.7	58.4
BMI (kg/m ²)	25.7	25.4	25.4	25.2	25.6
Weight gain from '86 to '90 (lbs)	1.1	1.5	1.6	1.5	1.9
Alcohol consumption in 1986 (g/day)	37.9	26.0	30.2	27.7	28.5
Alcohol consumption in 1990 (g/day)	16.4	21.2	30.1	32.2	49.2
Physical activity (METs/week)	39.5	40.2	38.1	39.8	39.7
Current smoker (%)	7.5	5.5	8.6	5.4	9.4
Family history of diabetes (%) ²	13.4	15.4	13.7	12.2	13.5
Cardiovascular disease (%) ³	11.4	10.9	10.3	9.5	8.8
Hypertension (%)	31.0	26.5	27.8	24.2	33.8
Hypercholesterolemia (%)	32.4	32.2	31.4	28.2	33.1

Abbreviations: BMI: body mass index (calculated as the weight in kilograms divided by height in meters squared; MET: metabolic equivalent; ellipses, not applicable.

¹ Data are given as mean for continuous variables, except alcohol consumption (median) and percentage for categorical variables and are age-adjusted, except for age.

² History of diabetes with onset at age ≥30 years in immediate family.

³ Cardiovascular disease comprises stroke, myocardial infarction, coronary artery bypass surgery and angina pectoris.

Table 2.2: Hazard ratios of type 2 diabetes according to updated 4-year changes in alcohol intake stratified by initial intake (N = 38,031)¹.

Initial alcohol consumption (g/day)	Change in alcohol consumption 4 years later (g/day)				
	Moderate to large decrease (≥7.5)	Small to moderate decrease (2.5–7.49)	No change / relatively stable (±2.49)	Small to moderate increase (2.5–7.49)	Moderate to large increase (≥7.5)
Non (0)					
Multivariable-adjusted ¹	1.00 (referent)	0.84 (0.55, 1.30)	0.63 (0.35, 1.13)
Person-years	89,008	5,170	3,229
No. of cases	448	22	13
<1 drink/day (0.1–14.9)					
Multivariable-adjusted ¹	1.22 (0.90, 1.66)	1.06 (0.89, 1.26)	1.00 (referent)	0.76 (0.63, 0.91)	0.84 (0.68, 1.03)
Person-years	7,446	33,804	113,714	42,162	26,600
No. of cases	45	165	534	145	112
≥1 drink/day (≥15.0)					
Multivariable-adjusted ¹	1.18 (0.91, 1.53)	1.14 (0.83, 1.56)	1.00 (referent)	1.04 (0.72, 1.51)	0.90 (0.66, 1.24)
Person-years	33,771	17,464	26,786	11,250	18,093
No. of cases	155	67	94	40	65
P_{interaction}²	0.004				

¹ Multivariable-adjusted hazard ratios (95% confidence intervals) were calculated using Cox proportional hazards model and adjusted for age (5 categories), body mass index (8 categories), physical activity (quintiles), smoking status (never, former, current 1–14 cigarettes/day, current 15–24 cigarettes/day or current ≥25 cigarettes/day), family history of type 2 diabetes, incident and prevalent cardiovascular disease (stroke, myocardial infarction, coronary artery bypass surgery or angina), hypertension, and hypercholesterolemia, dietary glycemic load (quintiles), fiber intake (quintiles), *trans* fat intake (quintiles), ratio of polyunsaturated fat and saturated fat (quintiles) (all energy adjusted), coffee intake (quintiles) and total energy intake (continuous).

² P_{interaction} was derived by adding an interaction term between the 7.5 g/day increment in alcohol consumption (continuous) and initial alcohol consumption (categorical) in the multivariate model. Ellipses, not applicable.

Concentrations of total adiponectin and HbA_{1c} per 7.5 g/day increment in alcohol intake over four years (i.e., 1990-1994) were calculated with a mixed analysis of variance model that included the same terms as the Cox regression model and a term for case-control status with clustering on case-control triads. The models were stratified by alcohol intake in 1990. For these analyses, we excluded men with missing data on alcohol intake and with a history of type 2 diabetes in 1994, leaving 697 and 1,188 men for the adiponectin and HbA_{1c} analyses, respectively.

Results

In 1990, the first update on alcohol consumption, most subjects (55%) reported only modest change in alcohol consumption compared to four years earlier (Table 2.1). The median change in alcohol consumption was 0 g/day. No consistent trends were seen in potential confounders among men who decreased or increased their alcohol consumption.

Overall alcohol consumption and risk of type 2 diabetes

During 428,497 person-years of follow-up among 38,031 men, we documented 1,905 cases of newly diagnosed type 2 diabetes. We first examined alcohol consumption in grams per day and risk of diabetes. Compared with abstinence, HRs of type 2 diabetes after multivariable adjustment were 1.04 (95% CI: 0.92, 1.18) for alcohol consumption of 0.1 to 4.9 g/day, 0.81 (95% CI: 0.69, 0.94) for 5 to 9.9 g/day, 0.70 (95% CI: 0.59, 0.84) for 10 to 14.9 g/day, 0.71 (95% CI: 0.60, 0.84) for 15 to 29.9 g/day, 0.54 (95% CI: 0.44, 0.67) for 30 to 49.9 g/day and 0.50 (95% CI: 0.36, 0.69) for alcohol consumption of 50 or more grams per day ($P_{\text{trend}} < 0.0001$).

Four-year changes in amount of alcohol intake and risk of type 2 diabetes

The effect of a 7.5 g/day (~half a glass) change in alcohol intake on subsequent change in diabetes risk differed between initial alcohol consumption levels ($P_{\text{interaction}} < 0.01$) (Table 2.2). Such an increment had the largest association with risk on men who were initially nondrinkers at the beginning of any four-year period of change (HR: 0.78; 95% CI: 0.60, 1.00). This was followed by men who initially consumed <1 glass/day (HR: 0.89; 95% CI: 0.83, 0.96). Four-year change in alcohol intake was not associated with subsequent diabetes risk among men who consumed ≥ 1 glass/day at the beginning of the four-year period (HR: 0.99; 95% CI: 0.95, 1.02). In a secondary analysis, we repeated the analysis excluding men with any missing alcohol data during follow-up. This slightly strengthened the association of changes in alcohol intake with risk among initial nondrinkers (HR: 0.71; 95% CI: 0.52, 0.98) but had little effect on initial <1 glass/day drinkers (HR: 0.89; 95% CI: 0.82, 0.96) and ≥ 1 glass/day drinkers (HR: 0.99; 95% CI: 0.95, 1.04). To minimize potential bias related to total abstinence from alcohol consumption due to poor health, we also excluded all current nondrinkers at the beginning of each follow-up period, with little effect on the HRs (data not shown). Finally, we examined whether changes in BMI and physical activity

over time could partially explain the observed relation. We assessed this effect by including levels of BMI and physical activity assessed at both the beginning and end of each four-year period used to calculate change in alcohol consumption, while retaining all other variables in the model. This did not materially influence our results (results not shown).

Table 2.3: Hazard ratios of type 2 diabetes according to updated 4-year changes in initial and current drinking category (N = 38,031).

Initial alcohol drinking category (g/day)	Alcohol drinking category four years later (g/day)			P_{trend}^I
	Light (0-4.9)	Moderate (5.0-29.9)	Heavier (≥30.0)	
Light (0-4.9)				
Hazard ratio ²	1.00 (referent)	0.75 (0.62, 0.90)	0.35 (0.11, 1.10)	< 0.001
Person-years	169,623	31,723	1,127	
No. of cases	875	125	3	
Moderate (5.0-29.9)				
Hazard ratio ²	1.09 (0.92, 1.30)	0.74 (0.65, 0.83)	0.59 (0.45, 0.77)	< 0.001
Person-years	27,841	134,942	16,050	
No. of cases	159	496	60	
Heavier (≥30.0)				
Hazard ratio ²	0.78 (0.44, 1.38)	0.67 (0.52, 0.88)	0.50 (0.40, 0.63)	0.08
Person-years	2,089	15,036	30,067	
No. of cases	12	65	110	

¹ P_{trend} was derived from tests of linear trend across increasing categories of alcohol use by treating the median value of each category as a continuous variable.

² Multivariable-adjusted hazard ratios (95% confidence intervals) were calculated using Cox proportional hazards model and adjusted for the covariates listed in table 2.

The association between changes in alcohol consumption and diabetes risk among the three strata of initial drinkers did not substantially differ by subgroups of BMI. The multivariable-adjusted relative risks for diabetes associated with a 7.5 g/day increase in alcohol among initial nondrinkers, <1 and ≥ 1 drink/day consumers were 0.66 (95% CI: 0.42-1.04), 0.85 (95% CI: 0.76, 0.94) and 0.98 (95% CI: 0.93, 1.04) for men with a BMI <28.3 kg/m² (median BMI of incident diabetes cases) and 0.86 (95% CI: 0.65, 1.14), 0.92 (95% CI: 0.84, 1.02) and 0.99 (95% CI: 0.93, 1.04) for men with a BMI of ≥ 28.3 kg/m², respectively ($P_{\text{interaction}} = 0.48$).

Table 2.4: Associations of a 7.5 g/day increment in alcohol consumption from 1990-1994 on subsequent total adiponectin (N = 697) and hemoglobin A_{1c} (HbA_{1c}) levels (N = 1,188), classified by alcohol consumption in 1990.

Alcohol consumption in 1990 (g/day)	Mean increment in glycemic marker per 7.5 g/day increase in alcohol consumption from 1990-1994	
	Total adiponectin (µg/mL)	HbA _{1c} (%)
Non (0)		
Mean increment ¹	1.2 ± 0.3	-0.04 ± 0.02
No. of participants	151	267
<1 drink/day (0.1-14.9)		
Mean increment ¹	0.5 ± 0.3	-0.02 ± 0.02
No. of participants	355	610
≥1 drink/day (≥15.0)		
Mean increment ¹	-0.6 ± 0.3	0.01 ± 0.01
No. of participants	191	311
<i>P</i> _{interaction} ²	0.002	0.02

¹ Values are mean increments (± SEM) per 7.5 g/day increment in alcohol consumption over four years and were calculated with a mixed analysis of variance model that included terms for age, body mass index, physical activity, smoking status, family history of type 2 diabetes, hypertension, hypercholesterolemia, cardiovascular disease, dietary glycemic load, fiber intake, *trans* fat intake, ratio of polyunsaturated fat and saturated fat (all energy-adjusted), coffee intake, total energy intake and case-control status.

² *P*_{interaction} was derived by adding an interaction term between the 7.5 g/day increment of alcohol consumption (continuous), and initial alcohol consumption in 1990 (categorical) in the mixed analysis of variance model.

Four-year changes in type of drinker and risk of type 2 diabetes

Compared to stable light drinkers (0-4.9 g/day), initial light drinkers who increased their intake to moderate levels (5.0-29.9 g/day) had a significantly lower risk of type 2 diabetes (HR: 0.75; 95% CI: 0.62, 0.90) (Table 2.3). Conversely, moderate drinkers who reduced their intake to none or light did not have a lower risk of diabetes (HR: 1.09; 95% CI: 0.92, 1.30) after multivariable adjustments compared to stable light drinkers. However, stable moderate drinkers had significantly lower risk of diabetes compared to stable light drinkers (HR: 0.74; 95% CI: 0.65, 0.83). Furthermore, all current moderate alcohol consumption categories were associated with at least a 25% lower risk of type 2 diabetes compared to stable light drinkers, regardless of initial alcohol consumption category (*P*_{interaction} = 0.55). No further risk reductions were observed among initial light or moderate drinkers who increased their consumption over ≥30.0 g/day. Similar results were obtained when we

reanalyzed the data excluding people who abstained from alcohol or who had missing alcohol data during follow-up (data not shown).

Four-year change in amount of alcohol intake and effect on markers of glycemia

To test the robustness of our findings, we next examined the four-year change in alcohol consumption from 1990-1994 on markers of glycemia collected in 1994, stratified by initial alcohol consumption in 1990 (Table 2.4). A similar interaction between change in alcohol and baseline alcohol intake as in the main analysis was observed for levels of total adiponectin and HbA_{1c} among non-diabetic men. For example, a 7.5 g/day increment in alcohol intake, between 1990-1994, was associated with a 1.2 ± 0.3 µg/mL (mean \pm SEM) higher adiponectin level in 1994 among men who were nondrinkers at baseline, a 0.5 ± 0.3 µg/mL higher adiponectin level among <1 glass/day drinkers, and a 0.6 ± 0.3 µg/mL lower level among ≥ 1 glass/day drinkers at baseline ($P_{\text{interaction}} = 0.002$). For HbA_{1c}, the inverse association between change in alcohol intake in 1990-1994 and HbA_{1c} concentration was also strongest among nondrinkers compared to <1 glass/day drinkers and ≥ 1 glass/day drinkers in 1990 ($P_{\text{interaction}} = 0.02$).

Discussion

In this large prospective cohort study, we found that four-year changes in alcohol consumption assessed repeatedly over time were followed by subsequent changes in risk of type 2 diabetes. The lower risk associated with an increase in alcohol consumption was dependent on initial drinking levels, with no benefit associated with increased intake among men already drinking moderately. This pattern of lower risk associated with increased alcohol consumption solely among abstainers and light drinkers was further supported by associations of change in alcohol intake with total adiponectin and HbA_{1c}.

Our results extend previous epidemiological studies that have reported an inverse association between moderate alcohol consumption and the longer term risk of type 2 diabetes (1;2). Recent studies have shown that alcohol consumption is associated with lower risk of type 2 diabetes even among low-risk individuals (lean, active non-smokers) (25;26) and when adjusted for multiple lifestyle factors based on BMI, physical activity level, smoking habits and diet quality (27-29). Comparisons of different beverage types generally suggest that ethanol rather than the type of alcoholic beverage is responsible for this association (23;30). Furthermore, variation in the *ADH1C* gene, a gene that encodes the alcohol dehydrogenase 1C enzyme that oxidizes ethanol, appears to modify the association between alcohol consumption and type 2 diabetes risk, providing further epidemiological support for the causal nature of the relationship between alcohol consumption and diabetes risk (31).

The plausibility of these observational results is supported by short-term randomized controlled trials on changes in alcohol consumption (25-30 g/day) (10;11). In these studies, moderate drinking significantly improved insulin sensitivity after six to eight

weeks. Also, clinical trials in a variety of populations have shown that alcohol consumption increases adiponectin, (11;12;32;33) a hormone secreted by adipose tissue that appears to improve insulin sensitivity. Indeed, adiponectin appears to explain approximately 25 to 30% of the inverse association between alcohol consumption and type 2 diabetes in women (34). Lastly, longer-term randomized trials of three to twelve months among diabetic individuals have shown that assignment to alcohol consumption lowers fasting glucose (35) and HbA_{1c} (36).

These findings from randomized trials suggest that the effects of alcohol intake on glycemia have a short latency, as they appear within weeks of assignment to alcohol. Our results are consistent with this finding, the more beneficial metabolic parameters and the lower subsequent risk of diabetes associated with an increase in alcohol consumption was evident in the next follow-up period. Our results further imply that the effect may be transient, as a decrease in consumption was accompanied by a modest increase in risk. Finally, our results highlight that any benefit of alcohol on glycemia and risk of diabetes is restricted to moderate drinking, and increases among those already drinking moderately confer no lower risk.

Several limitations warrant consideration. We relied on self-reported alcohol consumption. However, validation studies in these health professionals comparing self-administered questionnaire with intake assessed by detailed diet records showed correlations above 0.8 and mean and standard deviation values almost identical by the two methods (18). Second, we do not know why men changed their intake. However, we restricted our analysis to men without history of diabetes and cancer and adjusted for cardiovascular disease, hypertension and hypercholesterolemia. Third, we do not know when during each four-year interval the change in alcohol consumption occurred, a limitation that reflects the fact that the administered FFQ specifically queries alcohol consumption in the previous year. Therefore, we cannot definitely evaluate whether the change in intake on type 2 diabetes risk is immediate. We do know however, that the change in alcohol preceded the diagnosis of diabetes. Fourth, we performed our analysis in male health professionals and results may therefore not be readily generalizable to other populations. However, within this homogenous group of highly educated men potential confounding due to social economic status is substantially reduced. Fifth, we could not evaluate changes in beverage types, given the more limited use of any particular beverage compared with total alcohol use. We have previously found that all beverage types were inversely associated with type 2 diabetes in this cohort (24). Finally, as with any observational study, unaccounted factors associated with changes in alcohol consumption and risk of type 2 diabetes may introduce an unknown degree of residual confounding, despite the substantial number of potentially confounding factors we included.

In conclusion, in this cohort of male health professionals increases in alcohol consumption over time were associated with correspondingly lower four-year risk of type 2 diabetes, although this association was limited to rare and light drinkers at baseline. This suggests that the effect of alcohol consumption on diabetes risk has a relatively short

latency time but may also be transient and reversible. Our results also highlight that individuals already consuming alcohol in moderation did not receive further benefit from increased consumption. Although our results might be seen as suggesting that some individuals should consider adopting regular and moderate intake of alcohol, our findings – even if proven to be causal - are limited to a single outcome of diabetes. Decisions and recommendations about changes in alcohol consumption should, as with alcohol consumption in general, consider the full range of risks and benefits to an individual, including the consistent harms to the individual and society of drinking that exceeds recommended limits.

Author contributions

MMJ, SEC, KJM, HFJH and EBR contributed to the design of the study. FBH and EBR acquired the data. MMJ, SEC, KJM and EBR analysed and interpreted the data. MMJ and KJM drafted the first manuscript. HFJH, FBH and EBR obtained funding. KJM, FBH, HFJH and EBR supervised the study. All authors critically reviewed the manuscript for important intellectual content and approved the final version. MMJ and HFJH receive partial funding (unrestricted) from the Dutch Foundation for Alcohol Research (SAR).

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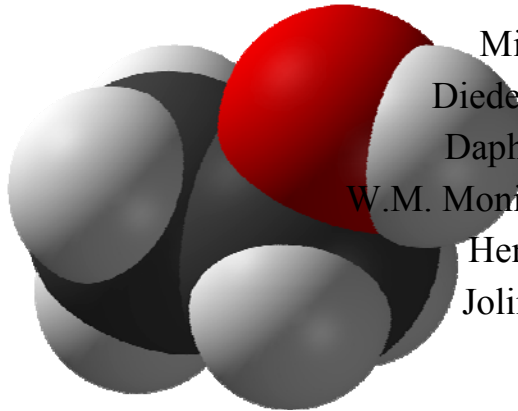
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Chapter 3

Combined effect of alcohol consumption and lifestyle behaviors on risk of type 2 diabetes



Michel M. Joosten
Diederick E. Grobbee
Daphne L. van der A
W.M. Monique Verschuren
Henk F.J. Hendriks
Joline W.J. Beulens

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Background: It has been suggested that the inverse association between alcohol and type 2 diabetes could be explained by moderate drinkers' healthier lifestyles.

Objective: We studied whether moderate alcohol consumption is associated with a lower risk of type 2 diabetes among adults with combined low-risk lifestyle behaviors.

Design: We prospectively examined 35,625 adults of the Dutch European Prospective Investigation into Cancer and Nutrition (EPIC)-NL cohort aged 20 - 70 years and free of diabetes, cardiovascular disease and cancer at baseline (1993-1997). Besides moderate alcohol consumption (women: 5.0-14.9 g/day; men: 5.0-29.9 g/day), we defined low-risk levels of four lifestyle behaviors: optimal weight (BMI <25 kg/m²), physically active (≥30 minutes of physical activity/day), current non-smoker and a healthy diet (upper two quintiles of the DASH diet).

Results: During a median of 10.3 years, we identified 796 incident cases of type 2 diabetes. Compared with teetotalers, hazard ratios of moderate alcohol consumers for risk of type 2 diabetes in low-risk lifestyle strata after multivariable adjustments were 0.35 (95% CI: 0.17, 0.72) when of a normal weight, 0.65 (0.46, 0.91) when physically active, 0.54 (0.41, 0.71) when nonsmoking and 0.57 (0.39, 0.84) when consuming a healthy diet. When ≥3 low-risk lifestyle behaviors were combined, the hazard ratio for incidence of type 2 diabetes among moderate alcohol consumers after multivariable adjustments was 0.56 (0.32, 1.00).

Conclusion: In subjects already at lower risk of type 2 diabetes on the basis of multiple low-risk lifestyle behaviors, moderate alcohol consumption was associated with an approximately 40% lower risk compared with abstention.

Introduction

Several studies have demonstrated that type 2 diabetes can largely be prevented through a healthy lifestyle (1;2). Components of such a lifestyle include maintaining a normal body weight (1), being physically active (3), refraining from smoking (4) and eating a healthy diet (5). Moderate alcohol consumption has consistently been associated with a decreased risk of type 2 diabetes in prospective cohort studies compared with abstinence or excessive consumption (6;7). Much of this association is thought to be attributable to an improved insulin sensitivity due to moderate alcohol consumption (8;9). Therefore, moderate alcohol consumption could be considered a fifth favorable behavioral lifestyle factor to lower the risk of type 2 diabetes.

Despite this evidence, some have suggested that the observed inverse association between alcohol and disease such as type 2 diabetes can be explained by the fact that moderate drinkers have a healthier lifestyle in general (10). Previous studies on the relation between alcohol consumption, lifestyle and type 2 diabetes have only considered individual lifestyle behaviors, (11-13) but low-risk lifestyle habits are often inter-correlated and may be most effective when present in combination.

Little is known about the association between type 2 diabetes risk and alcohol consumption in the context of healthy lifestyle behaviors, especially when multiple low-risk lifestyle habits are combined. We therefore examined the association of alcohol intake with risk of type 2 diabetes in strata of separate lifestyle behaviors based on body mass index (BMI), physical activity level, smoking and adherence to the Dietary Approach to Stop Hypertension (DASH) diet and in strata of combined low-risk lifestyle behaviors in a prospective population-based study of men and women (of which almost half were postmenopausal at baseline).

Methods

Study population

The EPIC-NL cohort is the Dutch contribution to the European Prospective Investigation into Cancer and Nutrition (EPIC) and consists of the Prospect and MORGEN cohort. Both cohorts were jointly set up within the context of EPIC between 1993 and 1997, recruiting predominantly Caucasian 40,011 subjects. This cohort is described in more detail elsewhere (14). In short, Prospect is a prospective cohort study of 17,357 women aged 49 - 70 who participated in the breast cancer screening between 1993 and 1997. The Monitoring Project on Risk Factors for Chronic Diseases (MORGEN)-cohort consists of 22,654 men and women aged 20 - 59 years who were recruited from three Dutch towns (Amsterdam, Doetinchem and Maastricht). From 1993 to 1997, each year a new random sample of ~5,000 subjects was examined. Of the total cohort of 40,011 persons, 615 subjects reported diabetes and 915 and 1,560 participants reported cardiovascular disease or cancer respectively at baseline and were therefore excluded. Furthermore, we excluded 192 subjects with missing data on alcohol consumption and diet, 138 persons with missing data

of other lifestyle behaviors, 36 individuals with unrealistic energy intakes (<600 kcal/day or >5000 kcal/day) and 930 individuals without consent for linkage to disease registries, leaving 35,625 subjects for the present study. The Prospect-EPIC study was approved by the Institutional Review Board of the University Medical Center Utrecht and the MORGEN project was approved by the Medical Ethical Committee of TNO, the Netherlands. All participants signed informed consent prior to study inclusion.

General assessments

At baseline, participants filled in a general questionnaire on demographic characteristics and risk behaviors for presence of chronic diseases. The general questionnaires from both cohorts were largely similar. Coding of this information was standardized and merged into one uniform database. Body weight, height, waist and hip circumferences were measured. Physical activity was assessed using a questionnaire, validated in an elderly population (15). The Cambridge Physical Activity Index (CPAI) was used to allocate individuals to four ordered categories: inactive, moderately inactive, moderately active and active, as previously described (16). Because we could not calculate a total physical activity score for 14% of all participants, we imputed missing scores by single imputation using linear regression modeling (SPSS MVA procedure) with other lifestyle factors (e.g. smoking, BMI) and the outcome (type 2 diabetes). Smoking was defined as never, past or current. Daily nutritional intake was obtained from a food frequency questionnaire (FFQ) containing questions on the usual frequency of consumption of 79 main food items during the year preceding enrolment. This questionnaire allows the estimation of the average daily consumption of 178 foods. The FFQ was validated against 12 monthly 24-hour recalls in a representative group before the start of the study (17;18).

We constructed the DASH diet score as previously described (19). The score is based on 8 components: high intake of fruits, vegetables (except potatoes and legumes), nuts and legumes, low-fat dairy products, and whole grains and low intake of sodium, sweetened beverages, and red and processed meats. The original DASH diet limits saturated fat intake, which would be in part reflected by a de-emphasis on red and processed meats in our score. For each of the components, we classified subjects into sex-specific quintiles according to their intake ranking. Component score for fruits, vegetables, nuts and legumes, low-fat dairy products, and whole grains is the sex-specific's quintile ranking. For example, quintile 1 is assigned 1 point and quintile 5, 5 points. For sodium, red and processed meats, and sweetened beverages, low intake was desired. Therefore, the lowest quintile was given a score of 5 points and the highest quintile, 1 point. We then summed up the component scores to obtain an overall DASH score ranging from 8 (lowest adherence) to 40 (highest adherence).

Education was categorized into three groups; low, average and high. Low education attainment included those with primary education up to those completing intermediate vocational education, average educational attainment included those with higher secondary education and high educational attainment included those with higher

vocational education and university. Women were assumed to be post-menopausal when they reported not having menstrual periods for at least a year. Serum HDL-cholesterol was measured in a 6.5% random sample of the baseline cohort using an automated enzymatic procedure on a Vitros 250 (Johnson & Johnson, Rochester, NY, U.S.A.).

Assessment of alcohol intake

Alcohol consumption was assessed by the general questionnaire and FFQ. The validity of this alcohol consumption assessment is good as confirmed by a Spearman correlation of 0.87 for women and 0.74 for men between the FFQ and twelve 24-hour recalls (17). Through the general questionnaire subjects were asked whether they had ever consumed alcohol. If so, they were asked the number of units of alcohol-free beer, beer, white wine, red wine, port/sherry/vermouth and spirits consumed. Subjects indicated their consumption frequency on a daily/weekly/monthly/yearly scale or as never consumed. Alcohol consumption at baseline was determined by multiplying the alcohol percentage of each beverage by the standard ethanol weight content (5% for beer, 18.5% for fortified wine, 12.5% for red wine, 12% for white wine and 40% for liquor; means from the average sorts of beverages).

Ascertainment of type 2 diabetes

The ascertainment of type 2 diabetes has been described in more detail elsewhere (20). The incidence of type 2 diabetes was first assessed by self-report in follow-up questionnaires and a urine dipstick test in the Prospect cohort only. Also data on diagnosis of type 2 diabetes were obtained from the Dutch Centre for Health Care Information, which holds a standardized computerized register of hospital discharge diagnoses [all diagnosis were coded according to the *International Classification of Diseases*, Ninth revision (ICD-9, ICD codes 250)] (21). The records from this database were linked to the EPIC-NL cohort with a validated probabilistic method (22). All potential cases of type 2 diabetes have been verified against medical records of the general practitioners and pharmacists. For 89% of all potential diabetes cases verification information was available. Of these potential diabetes cases 72% were verified as having type 2 diabetes and were used as such in the analysis. The remaining 28% was verified as not having type 2 diabetes or having another type of diabetes like gestational diabetes and were therefore included in our analysis as non-cases.

Definition of low-risk lifestyle behaviors and alcohol categories

We used the World Health Organization cut-off for healthy weight ($\text{BMI} < 25 \text{ kg/m}^2$). Results were essentially the same when we defined low risk for BMI as 18.5 to $< 25 \text{ kg/m}^2$ (data not shown). We used waist circumference as a surrogate measure for healthy weight. Low risk level was defined as a waist circumference of less than 88 cm in women or 92 cm in men (Adult Treatment Panel III criteria) (23). We dichotomized the population based on the four categories of CPAI into physically active (≥ 30 minutes of combined recreational, household, and occupational physical activity/day; CPAI categories: moderately active,

active) and physically inactive (<30 minutes of combined daily recreational, household, and occupational physical activity/day; CPAI categories: inactive, moderately inactive). In terms of cigarette smoking participants were categorized in current non-smokers (never/former) or current smokers. For the DASH diet score categories were: low adherence (lower 3 quintiles; composite score range of 8 to 25), or high adherence (upper 2 quintiles; composite score range of 26 to 40). Alcohol categories were divided in four alcohol groups in line with the 2005 U.S. DA Dietary Guidelines (24) as follows: teetotalers (lifelong abstainers), light (0-4.9 g/day for both women and men; includes former drinkers), moderate (between 5.0 to 14.9 g/day for women; 5.0 to 29.9 g/day for men) and heavier consumers (≥ 15.0 g/day for women; ≥ 30.0 g/day for men).

Statistical analysis

Follow up time was calculated from date of enrolment into the study to the date of diabetes diagnosis or date of death, all other participants were censored at the end of follow up (January 2006). We used Cox proportional hazards model to calculate relative risk for each alcohol consumption category using teetotalers as a reference and 95% confidence intervals (95% CI), adjusted for age (continuous), BMI, (continuous), physical activity (inactive; moderately inactive; moderately active; active), smoking (never, former and current), adherence to the DASH diet (quintiles), parental history of type 2 diabetes (present or not), education (high, middle, low), postmenopausal status (premenopausal, postmenopausal, perimenopausal and surgical postmenopausal), cohort (Prospect-EPIC-NL or MORGEN-EPIC-NL) and daily energy intake (continuous) and stratified by gender. To determine effects of alcohol consumption and individual lifestyle behaviors, the association of alcohol consumption with type 2 diabetes was first estimated stratified by each lifestyle factor separately. Secondly, to determine the effects of alcohol consumption and combined low-risk lifestyle factors on type 2 diabetes incidence, participants could score one point for each low-risk lifestyle: BMI <25 kg/m², physically active, current non-smoker, and upper two quintiles of the DASH diet. They could have a score ranging from 0 (no low-risk behaviors) to 4 (all low-risk behaviors). Statistical interaction of alcohol consumption with gender and with lifestyle behaviors was assessed based on likelihood ratio tests in models with and with out the cross-product terms in the multivariable model. Statistical analyses were performed using the SAS statistical software package (SAS version 9, SAS Institute, Cary, NC, U.S.A.).

Results

In both men and women, teetotalers smoked less and were less highly educated compared to alcohol consumers (Table 3.1). Less than 2.0% of the women reported drinking >45 g/day (>3 drinks/day) and 2.2% of the men reported drinking >75 g/day (>5 drinks/day). To validate the self-reported alcohol intake, we determined the relation between alcohol intake and HDL-cholesterol in a 6.5% sub-cohort. This analysis showed a linear association ($P <$

0.001) between alcohol intake and HDL-cholesterol concentrations in both men ($N = 604$) and women ($N = 1,719$) ($\beta \pm \text{SD}$: 0.062 ± 0.005 mmol/l per 5 g alcohol/day in women and 0.043 ± 0.007 mmol/l per 10 g alcohol/day in men) adjusted for all confounders from the multivariable model.

During a median follow-up of 10.3 person-years (360,661 person-years) we verified 618 female and 178 male incident cases of type 2 diabetes. Compared with female teetotalers hazard ratios (HRs) for type 2 diabetes were 0.75 (95% confidence interval (CI): 0.61, 0.91) for light, 0.48 (95% CI: 0.36, 0.63) for moderate and 0.54 (95% CI: 0.40, 0.73) for heavier drinkers after multivariable adjustment. Among males, HRs were 0.98 (95% CI: 0.53, 1.80) for light, 0.79 (95% CI: 0.43, 1.44) for moderate and 0.71 (95% CI: 0.37, 1.38) for heavier drinkers compared with male teetotalers after multivariable adjustment. Because we did not find a gender-based difference ($P_{\text{interaction}} = 0.50$) between alcohol consumption and type 2 diabetes, we combined men and women in further analyses. In the entire population, HRs for type 2 diabetes were 0.78 (95% CI: 0.64, 0.95) for light, 0.55 (95% CI: 0.43, 0.69) for moderate and 0.57 (95% CI: 0.44, 0.74) for heavier alcohol consumers compared with teetotalers after multivariable adjustment.

Prevalence of low-risk lifestyle behaviors was, 47.1% for having an optimal weight, 68.7% for having an optimal waist circumference, 62.3% for being physically active, 69.3 for not smoking and 38.5% for having a high diet quality. After multivariable adjustments, HRs for risk of type 2 diabetes in high-risk strata of lifestyle behaviors were 6.11 (95% CI: 4.74, 7.83) for BMI, 5.96 (95% CI: 5.02, 7.08) for waist circumference, 1.06 (95% CI: 0.92, 1.22) for physical activity, 1.19 (95% CI: 1.01, 1.40) for smoking and 1.18 (95% CI: 1.01, 1.37) for the DASH diet compared to the low-risk levels of each lifestyle behavior. When analyzing physical activity over the four categories in the multivariable adjusted model more pronounced associations were observed: Each increase in physical activity category was significantly associated with a 7% lower risk of type 2 diabetes after multivariable adjustments ($P_{\text{trend}} < 0.05$). Results were essentially the same when we excluded imputed values for physical activity.

Compared with teetotalers, HRs of moderate alcohol consumers for risk of type 2 diabetes in low-risk lifestyle strata after multivariable adjustments were 0.35 (95% CI: 0.17, 0.72) when having an optimal weight or 0.45 (95% CI: 0.28, 0.72) when having an optimal waist circumference, 0.65 (95% CI: 0.46, 0.91) when being physical active, 0.54 (95% CI: 0.41, 0.71) when being a non-smoker and 0.57 (95% CI: 0.39, 0.84) when consuming a healthy diet (Table 3.2). Results were essentially the same when we excluded imputed values for physical activity: 0.67 (95% CI: 0.46, 0.98). Comparable results were found for moderate alcohol consumers in subgroups reporting less healthy lifestyle behaviors.

Table 3.1: Distribution of variables by alcohol consumption of 26,243 Dutch women and 9,382 men of the Dutch European Prospective Investigation into Cancer and Nutrition (EPIC)-NL cohort at baseline¹.

	Women				Men			
	Alcohol consumption category (g/day)				Alcohol consumption category (g/day)			
	Teetotaler (0)	Light (0.0-4.9)	Moderate (5.0-14.9)	Heavier (≥15.0)	Teetotaler (0)	Light (0.0-4.9)	Moderate (5.0-14.9)	Heavier (≥15.0)
No. of participants (N)	3,370	11,461	6176	5,236	573	2,313	4,582	1,914
Age (years) ²	50±13	51±12	50±11	52±9	41±12	42±11	42±11	45±10
Body mass index (kg/m ²) ²	26.5±4.7	25.8±4.3	25.0±3.7	25.6±3.4	25.8±3.6	25.7±3.7	25.6±3.4	25.9±3.6
Waist circumference (cm) ²	84±12	83±11	81±10	82±10	92±11	92±11	92±10	94±11
Physically active ³ (%) ²	55.9	63.6	69.0	64.2	51.3	55.8	60.7	54.4
Current smokers (%) ²	22.9	23.5	28.4	40.2	29.1	33.0	36.5	53.3
Parental history of diabetes (%) ²	20.3	20.0	18.6	17.6	16.4	15.3	13.7	14.0
Postmenopausal (%) ²	45.1	47.0	42.9	47.3	-	-	-	-
High education level ⁴ (%) ²	13.2	22.7	37.1	41.7	23.6	29.7	42.5	41.7
Total energy intake (kcal/d) ²	1840±539	1838±481	1908±466	1955±470	2420±786	2500±690	2620±673	2775±719
Alcohol intake (g/day) ²	0 (0-0)	1 (0-3)	9.1 (7-12)	24 (20-33)	0 (0-0)	2 (1-3)	14 (9-21)	43 (35-56)
Components of DASH ⁵ diet								
Fruits (g/day) ²	280±182	297±174	291±167	269±164	253±200	240±189	222±160	197±172
Vegetables (g/day) ²	141±59	146±56	149±55	152±56	137±67	133±55	132±52	130±55
Nuts and legumes (g/day) ²	16±16	17±15	19±16	19±16	23±21	23±19	25±21	26±25
Whole grains (g/day) ²	53±60	63±59	69±57	66±53	62±86	73±92	85±89	70±77
Low-fat dairy intake (g/day) ²	269±223	290±220	289±213	263±212	261±257	261±251	260±237	212±217
Red and processed meat (g/day) ²	78±46	77±43	78±42	84±44	102±65	117±61	120±56	128±59
Sweetened beverages (g/day) ²	62±93	55±84	50±76	45±72	146±179	130±165	104±132	104±149
Sodium intake (mg/d) ²	2161±749	2171±656	2234±646	2201±698	2774±1126	2988±1043	3103±971	3024±1037

¹ Data are mean \pm SD or median (25th to 75th percentiles) for continuous variables or percent for dichotomous and categorical variables.

² *P* values < 0.05 between alcohol categories within gender based on ANOVA (continuous variables) or chi square (categorical variables).

³ Physical activity was based on an index of combined recreational, household, and occupational physical activity (Physically active: ≥ 30 minutes of combined recreational, household, and occupational physical activity/day).

⁴ High educational level: higher vocational education and university.

⁵ Dietary Approach to Stop Hypertension (DASH) diet is a diet focusing on 8 components: high intake of fruits, vegetables, nuts and legumes, low fat dairy products, and whole grains and low intake of sodium, sweetened beverages, and red and processed meats.

Table 3.2: Hazard ratios (95% CIs) for the risk of type 2 diabetes among 35,625 Dutch women and men according to alcohol consumption stratified by levels of Body Mass Index (BMI), waist circumference, physical activity, smoking and DASH diet score¹.

Lifestyle behavior	Alcohol consumption category (g/day)				P_{trend}^2	$P_{\text{interaction}}^3$
	Teetotaler (0, both men and women)	Light (0.0-4.9, both men and women)	Moderate (5.0-14.9, women; 5.0-29.9, men)	Heavier (≥15.0, women; ≥30.0, men)		
Body mass index (kg/m ²)						
<25	1.00 (referent)	0.44 (0.23, 0.84)	0.35 (0.17, 0.72)	0.43 (0.21, 0.89)	0.23	
No. of cases	15	27	15	15		
≥25	4.36 (2.56, 7.44)	3.38 (2.01, 5.67)	2.22 (1.30, 3.80)	2.22 (1.28, 3.84)	< 0.001	
No. of cases	138	343	144	101		0.38
Waist circumference (cm)						
<88 for women, <92 for men	1.00 (referent)	0.61 (0.41, 0.92)	0.45 (0.28, 0.72)	0.48 (0.29, 0.79)	0.05	
No. of cases	34	80	36	31		
≥88 for women, ≥92 for men	5.02 (3.42, 7.36)	4.04 (2.83, 5.78)	2.65 (1.80, 3.92)	2.59 (1.73, 3.90)	< 0.001	
No. of cases	119	288	123	85		0.65
Physical activity ⁴						
Physically active	1.00 (referent)	1.07 (0.80, 1.42)	0.65 (0.46, 0.91)	0.80 (0.56, 1.15)	0.02	
No. of cases	61	221	88	70		
Physically inactive	1.63 (1.18, 2.26)	0.94 (0.69, 1.27)	0.79 (0.55, 1.12)	0.67 (0.45, 1.00)	< 0.001	
No. of cases	92	147	71	46		0.01

Smoking						
Currently non-smoking	1.00 (referent)	0.78 (0.62, 0.96)	0.54 (0.41, 0.71)	0.73 (0.54, 0.98)	0.001	
No. of cases	121	278	104	80		
Smoking	1.21 (0.82, 1.80)	1.03 (0.78, 1.36)	0.75 (0.53, 1.04)	0.51 (0.35, 0.74)	< 0.001	
No. of cases	32	90	55	36		0.03
DASH⁵ diet						
High adherence	1.00 (referent)	0.81 (0.59, 1.13)	0.57 (0.39, 0.84)	0.45 (0.28, 0.73)	0.001	
No. of cases	49	142	59	29		
Low adherence	1.18 (0.84, 1.67)	0.89 (0.65, 1.22)	0.63 (0.44, 0.90)	0.74 (0.51, 1.07)	< 0.001	
No. of cases	104	226	100	87		0.38

¹ Hazard ratios were derived using Cox proportional hazard models and were adjusted for age (continuous), parental history of diabetes (present or not), menopausal status (premenopausal, surgical postmenopausal, perimenopausal and postmenopausal), education (high, middle, and low), cohort (Prospect or Monitoring Project on Risk Factors for Chronic Diseases), and energy intake/day (continuous) and were stratified by sex. Furthermore, models were adjusted for each of the other three lifestyle behaviors not stratified on: BMI (continuous), physical activity (inactive, moderately inactive, moderately active and active), smoking (never, former, current), and adherence to the DASH diet (quintiles).

² Derived using linear trend test across ordinal categories of alcohol consumption (teetotaler, light, moderate, heavier).

³ Derived using likelihood ratio tests from models with and with out the cross-product term of alcohol category (teetotaler, light, moderate, heavier) and each lifestyle behavior (low-risk, high-risk) in the multivariable model.

⁴ Physical activity was based on an index of combined recreational, household, and occupational physical activity (Physically active: ≥30 minutes of combined recreational, household, and occupational physical activity/day; physically inactive: <30 minutes of combined recreational, household, and occupational physical activity/day).

⁵ Dietary Approach to Stop Hypertension (DASH) diet is a diet focusing on 8 components: high intake of fruits, vegetables, nuts and legumes, low fat dairy products, and whole grains and low intake of sodium, sweetened beverages, and red and processed meats (high adherence: upper 2 quintiles (composite score range of 26 to 40); low adherence: lower 3 quintiles (composite score range of 8 to 25)).

Table 3.3: Hazard ratios (95% CIs) of type 2 diabetes according to drinking habits combined with 3 or 4, 2, and none or 1 low-risk lifestyle behaviors¹.

Number of low-risk lifestyle behaviors combined ²	Alcohol consumption category (g/day)				P_{trend} ³	$P_{\text{interaction}}$ ⁴
	Teetotaler (0, both men and women)	Light (0.0-4.9, both men and women)	Moderate (5.0-14.9, women; 5.0-29.9, men)	Heavier (≥15.0, women; ≥30.0, men)		
3 or 4	1.00 (referent)	1.13 (0.70, 1.83)	0.56 (0.32, 1.00)	0.63 (0.34, 1.18)	0.02	
No. of cases	21	92	30	20		
2	2.68 (1.62, 4.45)	1.70 (1.06, 2.72)	1.21 (0.73, 2.01)	1.43 (0.85, 2.42)	0.002	
No. of cases	61	131	61	48		
1 or none	3.90 (2.37, 6.42)	2.75 (1.72, 4.39)	1.98 (1.20, 3.28)	1.50 (0.89, 2.53)	< 0.001	
No. of cases	71	145	68	48		0.17

¹ Hazard ratios were derived by using Cox proportional hazards models and were adjusted for age (continuous), parental history of diabetes (present or not), menopausal status (premenopausal, postmenopausal, perimenopausal and surgical postmenopausal), education (high, middle, and low), cohort (Prospect or Monitoring Project on Risk Factors for Chronic Diseases), and energy intake/day (continuous) and stratified by sex.

² The four low-risk behaviors are: body mass index <25 kg/m², physically active (≥30 minutes of combined recreational, household, and occupational physical activity/day), currently not smoking (former and never smokers) and high adherence (upper two quintiles: composite score range of 26 to 40) to the DASH diet (a diet focusing on 8 components: high intake of fruits, vegetables, nuts and legumes, low fat dairy products, and whole grains and low intake of sodium, sweetened beverages, and red and processed meats).

³ Derived using linear trend test across ordinal categories of alcohol consumption (teetotaler, light, moderate, heavier).

⁴ Derived using a likelihood ratio test from models with and without the cross-product term of alcohol category (teetotaler, light, moderate, heavier) and combined low-risk lifestyle category (3 or 4, 2, and none or 1 low-risk lifestyle behaviors) in the multivariable model.

There were significant interactions between alcohol consumption categories and physical activity and between alcohol consumption categories and smoking, but these were mainly due to the lower risks of heavier drinkers who smoke or are physically inactive. Interactions between alcohol consumption categories and other dichotomous lifestyle behaviors were not significant (Table 3.2).

In order to have adequate power to investigate the impact of combined low-risk lifestyle factors across categories of alcohol consumption, we grouped participants with three or all four (38.2%), two (35.5%), and one or none (26.3%) low-risk lifestyle behaviors together. When ≥ 3 low-risk lifestyle behaviors were combined, HR for incidence of type 2 diabetes among moderate alcohol consumers after multivariable adjustments was 0.56 (95% CI: 0.32, 1.00) (Table 3.3). Results were essentially the same but more precise when we used the low-risk level for waist circumference instead of the low-risk level of BMI with the combined lifestyles analysis: 0.60 (95% CI: 0.37, 0.97). Similar associations were found among moderate alcohol consumers reporting less low-risk lifestyle habits. We observed no effect modification of the association of gender with combined low-risk lifestyle behaviors ($P_{\text{interaction}} = 0.23$) or alcohol and combined low-risk lifestyle behaviors ($P_{\text{interaction}} = 0.17$) after multivariable adjustment.

When analyzing data for each cohort separately almost identical HRs, though less precise, were observed among moderate alcohol consumers with ≥ 3 low-risk lifestyle behaviors combined. The HR for the Prospect cohort was 0.54 (95% CI: 0.27, 1.08) whereas the HR for the MORGEN cohort was 0.59 (95% CI: 0.22, 1.57) after multivariable adjustment. Again, when low-risk level of BMI was replaced with the low-risk level of waist circumference in the combined score, virtually the same HRs were observed among moderate alcohol consumers with ≥ 3 low-risk lifestyle behaviors combined when results from the multivariate model were split up according to cohort: Prospect cohort: 0.55 (95% CI: 0.30, 1.00); MORGEN cohort: 0.61 (95% CI: 0.28, 1.32).

Discussion

This prospective study shows that moderate alcohol consumption is associated with a reduced risk of developing type 2 diabetes, regardless of other favorable behavioral lifestyles. Among participants already at lower risk of developing type 2 diabetes based on the combined effect of body weight, physical activity level, smoking habits, and diet quality, moderate alcohol consumption was associated with a lower risk for the incidence of type 2 diabetes.

Our results confirm that moderate alcohol consumption reduces the risk of type 2 diabetes in the presence of individual low-risk lifestyle behaviors, such as BMI, physical activity and smoking, which is in line with 3 previous observational studies (11-13). In addition, they show that moderate alcohol consumption remains to be associated with a lower risk of type 2 diabetes among those with multiple low-risk lifestyle behaviors combined. These findings indicate that the relation between alcohol consumption and type

2 diabetes is not likely to be explained by a healthier lifestyle of moderate drinkers in general.

Our findings of joint moderate alcohol consumption and healthy lifestyle behaviors are comparable with findings of Mukamal *et al* for risk of coronary heart disease (25). In their study, men with 4 healthy lifestyle behaviors, who drank alcohol in moderation (5.0 to 29.9 g/day) remained to have a significant lower risk for myocardial infarction as compared with abstainers. Therefore, the associations of alcohol consumption with both type 2 diabetes and coronary heart disease do not seem to be confounded by healthier lifestyles of moderate drinkers.

In our cohort, heavier drinkers had lower risks of developing type 2 diabetes compared with teetotalers. Even heavier drinkers with a less favorable lifestyle behavior (based on smoking status, physical activity, and diet), had lower risks of developing type 2 diabetes compared with teetotalers with the favorable lifestyle behavior (Table 2). These findings warrant clarification. A reduction in the risk of developing type 2 diabetes is observed for an alcohol intake up to 50 g/day (6;7). However, the upper limit for moderate drinking in the current study was based on the 2005 U.S. DA Dietary Guidelines (24), directed at prevention of all chronic diseases rather than on maximum risk reduction of type 2 diabetes. These relatively low cutoffs, particularly for women, could explain the low-risks observed among the heavier drinkers.

Clearly, the observations for heavier drinkers must be considered in the light of potential harmful effects of excessive alcohol intake and greater risk of certain cancers such as breast and rectum (26). Heavy or excessive drinking should always be discouraged, whereas moderate alcohol consumption could be regarded as a complement, rather than an alternative, to other low-risk lifestyle habits. Guidelines and personal advice on alcohol consumption should consider the full range of benefits and risks to the individual.

The association between alcohol consumption and type 2 diabetes tended to be stronger in women than in men, which is consistent with previous meta-analyses (6;7). However, studies in these meta-analyses that reported results for both men and women did not find this trend. Men in this cohort were substantially younger than women and only about one-fourth of the entire cohort consisted of men. Subsequently, there were fewer incident cases of type 2 diabetes in men than in women, which may explain the weaker association between alcohol intake and incidence type 2 diabetes in men.

There are several biologically plausible mechanisms to explain an inverse association between moderate alcohol consumption and type 2 diabetes. In 2 short-term randomized controlled crossover trials of women without diabetes, alcohol consumption of 30 and 25 g/day decreased insulin resistance and triglycerides concentrations (8;9). Besides these effects, moderate alcohol consumption increases both gene expression and circulating protein levels of adiponectin (9). Adiponectin is positively associated with insulin sensitivity and inversely associated with inflammation and type 2 diabetes (27). Recently it has been suggested that adiponectin may explain about 25 to 30% of the inverse association between alcohol consumption and type 2 diabetes in women (28).

Some limitations of our study warrant consideration. First, a FFQ was used for nutritional assessment including alcohol intake. Self-reported alcohol intake is generally being underreported (29). However, there was a high correlation between alcohol consumption from the FFQ and the twelve 24-hour recalls and between alcohol consumption and serum HDL-cholesterol levels. This suggests sufficient validity for ranking participants on alcohol consumption. Also, we could not take drinking pattern (regular versus episodic/binge drinking) into account. However, among moderately drinking middle-aged and older women, binge drinking does not occur frequently (30). It is therefore unlikely that this influenced the results in this cohort of predominantly middle-aged and older women. Furthermore, we did not take into account beverage type in the association between alcohol and type 2 diabetes. However, a previous study in one of the cohorts presented here showed that beverage type did not influence this association (20).

Second, we used only baseline measurements to characterize individuals and did not take into account possible changes in alcohol consumption or other lifestyle behaviors over follow-up. People who are already ill or have poor health might be more likely to change their diet and could therefore drink less or even quit drinking as a result of this. However, we repeated the analyses excluding people with type 2 diabetes within the first two years of follow-up and the association persisted (data not shown). We excluded those with prevalent cardiovascular diseases and cancer at baseline. In addition, we used lifetime abstainers as a reference category without occasional or former drinkers to rule out “the sick quitter” hypothesis (31). Therefore, our results are unlikely to be confounded by such reverse causality.

Third, although we adjusted for several known potential confounders, we cannot completely rule out the possibility of residual confounding. Finally, presence of type 2 diabetes goes often undetected, and may be preclinical up to nine to twelve years (32). Undetected diabetes cases in our cohort may have been misclassified as nondiabetic individuals, which resulted in the attenuation of associations.

In summary, this study supports the presence of an inverse association between moderate alcohol consumption and incidence of type 2 diabetes in a mixed population and extends this association to subjects already at low-risk based on their multiple low-risk lifestyle behaviors. Moderate alcohol consumption (approximately 0.5 to 1 drink/day for women and 0.5 to 2 drinks/day for men) appears to reduce the risk of type 2 diabetes, independently of other lifestyle behaviors.

Authors' contributions

The authors' responsibilities were as follows - MMJ, HFJH, and JWJB: concept and design of the study; DEG, DLA, WMMV, and JWJB: acquisition of the data; MMJ, and JWJB: conducting the statistical analyses and writing of the first draft of the manuscript. DEG, DLA, WMMV, and HFJH: critical review, advice and consultation throughout. MMJ and HFJH receive partial funding (unrestricted) from the SAR, a Dutch organization for alcohol research. DEG, DLA, WMMV, and JWJB report no conflict of interest.

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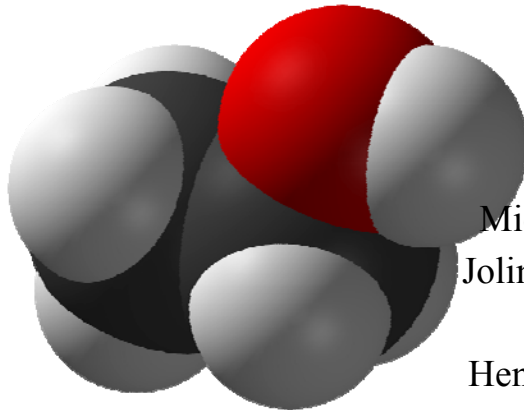
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Chapter 4

Moderate alcohol consumption increases insulin sensitivity and *ADIPOQ* gene expression in postmenopausal women: a randomized, crossover trial



Michel M. Joosten
Joline W.J. Beulens
Sander Kersten
Henk F.J. Hendriks

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Background: Moderate alcohol consumption has consistently been associated with a decreased risk of type 2 diabetes compared with abstaining and excessive drinking but whether it improves insulin sensitivity remains uncertain.

Objective: To determine whether six weeks of daily, moderate alcohol consumption increases expression of the gene encoding adiponectin (*ADIPOQ*) and plasma levels of the protein, and improves insulin sensitivity in postmenopausal women.

Methods: In a randomized, open-label, crossover trial conducted in the Netherlands, 36 apparently healthy postmenopausal women who were regular alcohol consumers, received 250 mL white wine (~25 g alcohol/day) or 250 mL of white grape juice (control) daily during dinner for six weeks. Randomization to treatment allocation occurred according to BMI. Insulin sensitivity and *ADIPOQ* mRNA and plasma adiponectin levels were measured at the end of both periods. Insulin sensitivity was estimated using the homeostasis model assessment of insulin resistance (HOMA-IR). Levels of *ADIPOQ* mRNA in subcutaneous adipose tissue were determined by RT-PCR.

Results: All participants completed the study. Six weeks of white wine consumption reduced fasting insulin (mean \pm SEM 40.0 ± 3.4 vs. 46.5 ± 3.4 pmol/L; $P < 0.01$) and HOMA-IR (1.42 ± 0.13 vs. 1.64 ± 0.13 ; $P = 0.02$) compared with 6 weeks of grape juice consumption. *ADIPOQ* mRNA levels (1.09 ± 0.15 vs. 0.98 ± 0.15 ; $P = 0.04$) and plasma levels of total (13.1 ± 0.8 vs. 12.0 ± 0.8 μ g/mL; $P < 0.001$) and high-molecular weight (HMW) adiponectin (9.9 ± 1.2 vs. 8.8 ± 1.2 μ g/mL; $P = 0.02$) significantly increased after alcohol consumption compared with juice consumption. Changes in *ADIPOQ* mRNA levels correlated with changes in plasma levels of total adiponectin ($\rho = 0.46$; $P < 0.01$). Both fasting triglyceride (8.2%; $P = 0.04$) and LDL-cholesterol levels (7.8%; $P < 0.0001$) decreased whereas HDL-cholesterol increased (7.0%; $P < 0.0001$) after prolonged moderate alcohol intake. No notable adverse events were reported.

Conclusions: Moderate alcohol consumption for six weeks improves insulin sensitivity, adiponectin levels and lipid profile in postmenopausal women. Furthermore, these data suggest a transcriptional mechanism leading to the alcohol-induced increase in adiponectin plasma levels.

Trial registration: Clinicaltrials.gov ID no.: NCT00285909.

Introduction

Moderate alcohol consumption has consistently been associated with a decreased risk of type 2 diabetes compared with abstaining and excessive drinking (1;2). The reason for the lower risk of type 2 diabetes is not entirely clear but a plausible explanation seems to be improved insulin sensitivity (3). However, so far only one randomized controlled trial among postmenopausal women has confirmed this notion (4). Other studies, mainly performed among young male populations, failed to detect such effects (5-12).

Adiponectin, an adipose tissue-derived hormone, is thought to play an important role in the regulation of insulin sensitivity and glucose and lipid metabolism (13). Its plasma levels are positively associated with insulin sensitivity (14) and are inversely associated with impaired glucose metabolism and type 2 diabetes (15-17). Furthermore, observational studies revealed an association between moderate alcohol consumption, adiponectin concentrations and insulin sensitivity (18;19). Randomized controlled trials also showed a significant increase of both circulating levels of total and high-molecular weight (HMW) adiponectin (8;11;12) after a period of moderate alcohol consumption compared with abstention.

However, the mechanism by which moderate alcohol consumption increases adiponectin levels is unknown. Synthesis of adiponectin is controlled by peroxisome proliferator-activated receptor γ (PPAR γ) activation, which functions as a transcriptional regulator (20). Low doses of alcohol have been shown to alter the expression of genes encoding tissue plasminogen activator (*PLAT*) and plasminogen activator inhibitor type-1 (PAI-1 also known as *SERPINE1*) (21). Like adiponectin, these markers are affected by moderate alcohol consumption and activated by PPAR γ (22).

Consequently, we investigated whether moderate alcohol consumption affects gene expression of the gene encoding adiponectin (*ADIPOQ*) and levels of the protein, and whether it improves insulin sensitivity in postmenopausal women.

Methods

Study protocol

The study used a randomized, open-label, placebo-controlled, crossover design, consisting of two 6-week periods, each preceded by a 1 week of washout. Participants consumed 250 mL of white wine (25 g alcohol; Chardonnay; Jean d'Alibert, Rieux, France) or alcohol-free white grape juice (Albert Heijn, Zaandam, the Netherlands) daily for 6 weeks during dinner. The study was conducted at TNO (a Dutch acronym for applied scientific research) Quality of Life, in Zeist, the Netherlands. The primary objective of the study was to investigate the effect of moderate alcohol consumption on PPAR γ activity and risk markers of metabolic disease and took place from March through June 2006. Participants were instructed to maintain their habitual body weight, food pattern and physical activity pattern and were told to refrain from any alcoholic products during the entire study (including washout periods) except for alcoholic beverages supplied by TNO. Of the 77 women who

were screened, 40 were eligible to participate. A total of 36 women were randomly selected and initiated the study. Allocation to treatment order was randomized according to BMI. Both white wine and white grape juice contained 300kJ/100mL (~70 kcal/100mL). Blood and adipose tissue sampling was done on the last day of each treatment period after an overnight fast. Compliance was monitored by weekly measurement of urinary ethyl glucuronide (EtG), a direct phase II metabolite of ethanol formed by action of UDP-glucuronosyl transferase. EtG has been reported to be a superior marker with 100% sensitivity as a biomarker of recent drinking (23). It persists in the urine up to ~75 - 85 h after last intake. Additional measures of compliance were increase of HDL-cholesterol, daily questionnaires, and return of empty bottles.

Participants

Postmenopausal women were recruited from a pool of volunteers of TNO Quality of Life and by advertisements in local newspapers. Eligible participants consumed between 5 and 21 units of alcohol per week, were apparently healthy and had an absence of menses for at least two years, a BMI between 18.5 and 35 kg/m² and no family history of alcoholism. Participants gave written informed consent and received a compensation for their participation. An independent medical ethical committee (The Medical Ethical Committee University Medical Centre Utrecht) approved the research protocol.

Outcomes measures

Primary endpoints were markers of insulin sensitivity such as homeostasis model assessment of insulin resistance (HOMA-IR), glucose, insulin, HbA_{1c} and free fatty acids (FFA) and several adiponectin-related measures such as *ADIPOQ* mRNA levels and plasma levels of total and HMW adiponectin. Secondary variables included lipid profile (triglycerides, HDL-and LDL-cholesterol) HbA_{1c} and FFA and correlations between specific outcome measures.

Analyses and assessment

Ethyl glucuronide was determined in morning urine samples. Urinary samples were diluted about twenty times using an internal standard solution. The resulting solution was analyzed using a triple quadrupole Ultra Performance LC/MS in MRM mode (Waters, Saint-Quentin En Yvelines Cedex, France) with a detection limit of 50 ng/mL. Cut-off limit to assess compliance during juice intervention for urinary EtG values was set at >0.25 mg/mL (positive sample), to obtain a high sensitivity but avoid positive results due to unintentional ethanol exposure. Cut-off limit during wine intervention was set at EtG values <0.5 mg/mL (2.2 µmol/L) (negative sample) as proposed by Bottcher et al. (24). Blood samples were obtained from the antecubital vein of the forearm and collected in tubes containing clot activator for serum and in ice-chilled tubes containing or Potassium Ethylene Diamine Tetra Acid (K₃EDTA) for plasma (Vacutainer Systems, Becton Dickinson, Plymouth, U.K.). Blood was centrifuged for 15 minutes at 2.000 g at 4°C, within 15 - 30 minutes after

collection and stored at -80°C . All biochemical determinations in blood were performed at TNO Quality of Life using Olympus analytical equipment and reagents except for adiponectin. Plasma total adiponectin concentrations were determined by radioimmunoassay (RIA) method (Linco Research, Inc., St. Charles, MO, U.S.A.) with a mean intra-assay coefficient of variation of 5.0% and plasma high molecular weight (HMW) adiponectin levels by a novel Enzyme-linked immunosorbent assay (ELISA) (Linco Research) with a mean intra-assay coefficient of variation of 14.1%. LDL-cholesterol was calculated by the Friedewald formula. Subcutaneous adipose tissue was sampled from the buttock by means of a Strauss cannula, outer diameter 1.5 mm and stored at -70°C until further analysis. Total RNA was extracted from adipose tissues with TRIzol reagent (Invitrogen, Breda, The Netherlands); 1 mg total RNA was then reverse-transcribed with iScript (Bio-Rad, Veenendaal, The Netherlands). cDNA was PCR-amplified with Platinum Taq DNA polymerase (Invitrogen) on a Bio-Rad iCycler or MyIQ PCR machine. Expression were standardized to levels of acidic ribosomal protein *ARBP* (25). Primer sequences used were *ADIPOQ* forward: TATCCCCAACATGCCCATTCG, *ADIPOQ* reverse: TGGTAGGCAAAGTAGTACAGCC, *PPARG* forward: TCCATGCTGTTATGGGT-GAA, *PPARG* reverse: TCAAAGGAGTGGGAGTGGTC, *ARBP* forward: CGGGAAGGCTGTGGTGCTG and *ARBP* reverse: GTGAACACAAAGCCCACA-TTCC.

Statistical analyses

Statistical analyses were performed using the SAS statistical software package (SAS version 8, SAS Institute, Cary, NC, U.S.A.). All variables were compared between treatments with a mixed analysis of variance model that included terms for treatment, period and the interaction between period and treatment (indicating possible carryover effects). Body weight was included in the model as a random factor to adjust for changes of body weight. Correlation coefficients were computed according to Spearman rank order to assess associations between intervention-induced changes in outcome measures. Based on a study by Arvidsson *et al.* (26) a sample size of 36 should be sufficient to detect a 20% difference in *ADIPOQ* mRNA levels with a power of 80% and accepting a two-sided α of 0.05. Data are presented as means and SEM unless otherwise specified. Statistical significance was defined as $P < 0.05$.

Results

General results

All 36 postmenopausal women completed the study. Participants were slightly overweight (BMI $25.4 \pm 3.3 \text{ kg/m}^2$). Mean glucose, insulin, triglycerides and cholesterol concentrations before the intervention indicated that participants were euglycemic and normolipidemic (Table 4.1).

Table 4.1: Characteristics of 36 postmenopausal women before intervention after an overnight fast.

Variable	Baseline
Age (years)	56.5 ± 4.2
Weight (kg)	71.4 ± 10.0
BMI (kg/m ²)	25.4 ± 3.3
Glucose (mmol/L)	5.5 ± 0.4
Insulin (pmol/L)	37.4 ± 12.6
Triglycerides (mmol/L)	1.21 ± 0.54
HDL-cholesterol (mmol/L)	1.67 ± 0.27
LDL-cholesterol (mmol/L)	3.88 ± 0.60
Total cholesterol (mmol/L)	6.11 ± 0.72
Alkaline phosphatase (U/L)	76.1 ± 19.3
Alanine aminotransferase (U/L)	15.3 ± 8.2
Aspartate aminotransferase (U/L)	20.8 ± 7.4
γ-Glutamyltranspeptidase (U/L)	21.3 ± 11.4
Follicle-stimulating hormone (IU/L)	108.5 ± 41.3

Data are means ± SD

Compliance with treatments was assessed by weekly urinary EtG revealing two positive samples during the juice-drinking period and six negative samples during the wine-drinking period out of the 468 samples analyzed (overall compliance of 98.3%). Another indicator for compliance was the 7.0% increase in HDL-cholesterol after wine compared with juice consumption (Table 4.2).

No significant ($P > 0.05$) carryover effects, as assessed by an interaction between treatment and period effect, were observed for any outcome measures. Treatment means of variables and P values estimated from models with period effects were very similar to those obtained from models without period effect, and we report the results of the latter. Mean body weight was 0.7 kg higher ($P < 0.001$) at the end of the alcohol drinking period (Table 4.2). Results were adjusted for this difference in body weight, but did not essentially change them. Therefore, unadjusted results are presented here.

Insulin sensitivity

Fasting serum insulin concentrations decreased (12.3%; $P < 0.01$) and HbA_{1c} percentages ($P < 0.09$) tended to decrease after six weeks of moderate alcohol consumption compared with abstinence. Insulin sensitivity, as expressed by the HOMA-IR index, also improved by 11.9 % ($P = 0.02$) after white wine consumption. However, there were no differences between serum glucose and free fatty acid concentrations after the two treatment periods (Table 4.2).

Table 4.2: Markers of insulin sensitivity, adiponectin and lipid profile in 36 postmenopausal women measured at the end of a 6 week period of consuming white grape juice or white wine after an overnight fast.

Variable	Grape juice	White wine	<i>P</i> value ¹
Markers of insulin sensitivity			
HOMA-IR score	1.64 ± 0.13	1.42 ± 0.13	0.02
Insulin (pmol/L)	46.5 ± 3.4	40.0 ± 3.4	< 0.01
Glucose (mmol/L)	5.4 ± 0.1	5.4 ± 0.1	0.72
HbA _{1c} (%)	6.0 ± 0.04	5.9 ± 0.04	0.09
Free fatty acids (mmol/L)	0.43 ± 0.04	0.44 ± 0.04	0.67
Weight (kg)	70.4 ± 1.7	71.1 ± 1.7	< 0.001
Adiponectin			
<i>ADIPOQ</i> / <i>ARBP</i> (arbitrary units)	0.98 ± 0.15	1.09 ± 0.15	0.04
<i>PPARG</i> / <i>ARBP</i> (arbitrary units)	0.67 ± 0.09	0.73 ± 0.09	0.13
Total adiponectin (µg/mL)	12.0 ± 0.8	13.1 ± 0.8	< 0.001
High-molecular weight adiponectin (µg/mL)	8.8 ± 1.2	9.9 ± 1.2	0.02
Lipid profile			
Triglycerides (mmol/L)	1.18 ± 0.08	1.03 ± 0.08	0.04
HDL-cholesterol (mmol/L)	1.57 ± 0.04	1.68 ± 0.04	< 0.0001
LDL-cholesterol (mmol/L)	3.84 ± 0.12	3.51 ± 0.12	< 0.0001

Data are means ± SEM.

¹ *P* values obtained from a mixed ANOVA model that included terms for treatment, period and the interaction between period and treatment (to assess carryover effects).

Adiponectin

There was a significant increase in the relative *ADIPOQ* mRNA levels in subcutaneous adipose tissue (27.7%; *P* = 0.04) after alcohol consumption compared with grape juice. Consistently, the ratio of *PPARG*/*ARBP* mRNA levels tended to increase as well although not significantly (*P* = 0.13) after the alcohol treatment. Both plasma protein levels of total adiponectin (10.6%; *P* < 0.001) and HMW adiponectin (15.8%; *P* = 0.02) increased significantly after consuming white wine, rather than juice, for 6 weeks (Table 4.2).

Lipid profile

Besides a significant increase in HDL-cholesterol, changes in lipid profile were further characterized by significant decreases in LDL-cholesterol (7.8%; *P* < 0.001) and triglycerides (8.2%; *P* = 0.04) levels after the wine-drinking period compared with the juice-drinking period (Table 4.2).

Correlations

Changes in *ADIPOQ* mRNA levels due to alcohol consumption correlated positively with changes in *PPARG* mRNA levels ($\rho = 0.76$; $P < 0.001$) and with changes in the plasma protein level of total adiponectin ($\rho = 0.46$; $P < 0.01$), but not with changes in plasma levels of HMW adiponectin ($\rho = -0.25$; $P = 0.15$). No significant correlations were found between changes in HOMA-IR and changes in total ($\rho = 0.27$; $P = 0.11$) or HMW ($\rho = -0.18$; $P = 0.29$) adiponectin plasma levels. Furthermore, changes in HDL-cholesterol correlated significantly with changes in total adiponectin ($\rho = 0.47$; $P < 0.01$) but not with HMW adiponectin levels ($\rho = 0.13$; $P = 0.44$).

Discussion

The primary findings of this study are that daily moderate alcohol intake improves insulin sensitivity and lipid profile in postmenopausal women. Furthermore, *ADIPOQ* mRNA levels in subcutaneous adipose tissue along with plasma levels of total and HMW adiponectin increased after moderate alcohol consumption. Moreover, changes in *ADIPOQ* mRNA levels correlated with changes in plasma total adiponectin indicating that prolonged moderate alcohol intake increases circulating adiponectin via increased gene expression. However, improvements in insulin sensitivity were not associated and with increased plasma adiponectin levels (HMW and total) after moderate alcohol consumption in these women.

A strength of this study is its randomized crossover design. In addition, we assessed compliance to study treatment several times in different ways throughout the study and observed no significant deviations. Several limitations warrant consideration. A slightly lower body weight was observed after the juice intervention compared with the alcohol intervention. However, lower body weight is associated with improved insulin sensitivity and thus could have only affected the results towards no alcohol-induced improvement in insulin sensitivity. In the present study, only subcutaneous adipose tissue samples were used to determine *ADIPOQ* mRNA levels. Studies have demonstrated that *ADIPOQ* mRNA levels were higher (27;28) or not different (29) in subcutaneous compared with visceral adipose tissue in lean and obese subjects. Furthermore, studies in human adipose tissue showed increased (30) as well as indifferent (31) *ADIPOQ* mRNA and *in vitro* protein secretion levels of adiponectin from subcutaneous compared with omental adipocytes. Subcutaneous adipose tissue thus, appears to be at least of equal importance as omental adipose tissue for circulating plasma adiponectin levels.

The observed changes in insulin sensitivity after prolonged moderate alcohol intake are in accordance with a study by Davies *et al.* (4) in which moderate alcohol consumption in postmenopausal women for 8 weeks decreased fasting insulin and triglycerides levels, both independent risk factors for type 2 diabetes, and improved insulin sensitivity. However, other clinical trials did not find a change in fasting insulin levels (5) and triglyceride levels (32;33) nor in insulin sensitivity after moderate alcohol consumption

(6;12), whereas levels of total adiponectin (8;10;12) and HMW adiponectin (11) did increase. Differences in the designs used in these clinical trials are numerous. However, studies that did observe an effect on markers of insulin sensitivity after moderate alcohol consumption were all performed in middle-aged (4), relatively insulin resistant (8) or diabetic subjects (34;35). This suggests that the effect of moderate alcohol intake on insulin sensitivity is more pronounced in subjects with (slightly) impaired glucose tolerance rather than young and glucose tolerant subjects. Moreover, most interventions that did not observe a link between alcohol and insulin sensitivity lasted only 30 (7;9) or even fewer (8;10-12) days of consecutive alcohol consumption, while this study and of Davies *et al.* (4) and Shai *et al.* (34) comprised of 6, 8 and 12 weeks respectively. This indicates that a longer duration of alcohol consumption may be needed to exert an effect on insulin sensitivity. Finally, the relation between moderate alcohol intake and insulin sensitivity might be influenced by gender since the association between alcohol consumption and type 2 diabetes appears to be stronger in women than men as observed in some observational studies (2). This gender difference may partly be explained by changes associated with menopause which may mediate the relation between alcohol and triglyceride levels, the latter being an independent risk factor for type 2 diabetes (36). Compared with postmenopausal non-drinkers, postmenopausal drinkers have lower triglyceride levels whereas male drinkers have higher triglyceride levels compared with male abstainers (37).

Intervention-associated changes in *ADIPOQ* mRNA levels in subcutaneous adipose tissue correlated with changes in plasma protein levels of total adiponectin. In line with are cross sectional studies (38;39) showing associations between subcutaneous *ADIPOQ* mRNA levels with plasma levels of adiponectin. This finding suggests that the alcohol-induced increase in plasma adiponectin levels is mediated by *de novo* synthesis rather than decreased renal function (40) or post-transcriptional regulation (41). Changes in plasma HMW adiponectin did not correlate with changes in *ADIPOQ* mRNA levels. However, multimer oligomer formation of HMW adiponectin is influenced by several post-translational modifications (42) which can account for the absence of such a correlation.

Adiponectin synthesis is under control of the transcription factor PPAR γ (20). PPAR γ ligands induce expression of the adiponectin gene via direct binding of the PPAR γ / retinoid X receptor heterodimer to the PPAR-responsive element in the human *ADIPOQ* promoter. We indeed observed a trend for increased *PPARG* mRNA levels after the alcohol intervention. However, our sample size of 36 participants was anticipated to detect a 20% change in mRNA levels. As the intervention gave rise to only a 9% change in *PPARG* mRNA, our study was underpowered to detect a significant difference in. Besides this trend for increased *PPARG* mRNA levels we also found a strong correlation between intervention-associated changes in *PPARG* and *ADIPOQ* mRNA levels. Furthermore, previous research has shown that *PAI-1* expression, and PAI-1 protein levels increases after alcohol consumption (21). *PAI-1* expression is also regulated by PPAR γ (22). It thus seems plausible that moderate alcohol use might alter adiponectin gene expression and consequently adiponectin protein levels, also in a PPAR γ -mediated manner.

Consistent with previous findings in young and middle-aged men (8;10), we now report that also in postmenopausal women total adiponectin plasma levels increase after moderate alcohol consumption. Moreover, we found that also the high-molecular weight form of plasma adiponectin increased after six weeks of alcohol consumption. The increase in HMW adiponectin after moderate alcohol consumption is in line with a previous study in which a trend was observed (11). In the current study, however, we used a more sensitive and precise methodology to quantify HMW adiponectin, ELISA instead of quantitative Western blotting.

The observed changes in insulin sensitivity as measured by HOMA-IR did not correlate with the alcohol-induced changes in total or with HMW adiponectin, of which the latter is proposed to be a better predictor of insulin sensitivity (43). These findings are consistent with other lifestyle interventions like weight loss (44) and exercise interventions (45) and with previous observations of our group (11;12) which showed increased adiponectin plasma levels without changes in insulin sensitivity. In this study we do find an alcohol-effect on insulin sensitivity irrespective of changes in adiponectin levels. The present results may suggest a possible independent effect of alcohol on insulin sensitivity in addition to an adiponectin-mediated effect on insulin sensitivity.

Looking cross sectional at our data, we did observe correlations between HOMA-IR and adiponectin levels after both alcohol and placebo treatment (data not shown). This is in line with published literature in which consistently associations between adiponectin and insulin sensitivity have been shown (14-17). Possibly, increased adiponectin levels partly explain the improved insulin sensitivity seen among moderate alcohol consumers (18;19). Studies have revealed that circulating adiponectin levels are inversely associated with markers of inflammation (39;46;47) whereas a low-grade inflammation is thought to contribute to insulin resistance (48). Therefore, adiponectin may lead to improved insulin sensitivity through effects on inflammatory markers.

In conclusion, the results of the present study demonstrate that six weeks of moderate alcohol consumption improves insulin sensitivity, and lipid profile in postmenopausal women. Furthermore, moderate alcohol consumption increases subcutaneous adipose *ADIPOQ* mRNA levels and plasma levels of adiponectin. This suggests that the alcohol-induced increase in adiponectin is attributable at least in part to transcriptional alterations in adipose tissue. The observed improvement in insulin sensitivity, lipid profile and adiponectin levels when consuming 2.5 standard drinks per day may reduce the risk of type 2 diabetes and cardiovascular disease in postmenopausal women. Despite these potentially beneficial findings, scientific literature indicates that alcohol drinking may also have detrimental health effects (49). Both potential benefits and risks should be taken into consideration when counseling on consumption.

Duality of interest

The authors declare that there is no duality of interest associated with this manuscript.

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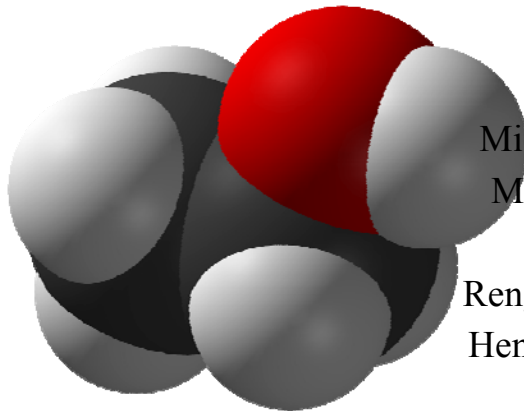
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Chapter 5

Moderate alcohol consumption alters leukocyte gene expression profiles and proteins related to immune response and lipid metabolism in men



Michel M. Joosten
Marjan J. van Erk
Linette Pellis
Renger F. Witkamp
Henk F.J. Hendriks

Submitted in revised form

Background: Moderate alcohol consumption has various acute and longer-term effects on low-grade inflammation which may accumulatively affect chronic disease risk. So far, no comprehensive, large-scale profiling has been performed to integrate the effects of longer-term moderate alcohol consumption.

Objective: To describe the effects of moderate alcohol consumption on several circulating markers and whole-genome transcriptomics using large-scale profiling of proteins and gene expression activity in blood and in blood cells, respectively.

Methods: In a randomized, open-label, crossover trial, 24 young (age: 21-33 y) normal-weight (BMI: 19-27 kg/m²) men consumed 100 mL vodka (30 g alcohol) with 200 mL orange juice or orange juice only (control) daily during dinner for four weeks. After each 4-week period, blood was sampled for measuring serum lipoproteins, plasma cytokines and whole blood gene expression. To determine gene expression profiles whole blood was collected in PAXgene tubes and extracted RNA was analyzed on Illumina BeadChips.

Results: Four weeks of vodka increased HDL-cholesterol, associated lipoprotein levels and total adiponectin whereas interleukin (IL)-1 receptor antagonist and IL-18 plasma levels decreased (all $P < 0.05$) compared with control. Four weeks of vodka resulted in down-regulation of 345 genes and up-regulation of 455 genes in blood cells. Pathway analysis revealed effects of vodka consumption on the immune response with a down-regulated expression of the nuclear transcription factor kappa B (NF- κ B) in blood cells. Furthermore, a network of expression changes related to lipid metabolism was observed with a central role for peroxisome proliferator-activated receptor alpha (PPAR α).

Conclusion: An integrated approach of large-scale profiling of proteins and genes showed that longer-term moderate alcohol consumption altered immune response and lipid metabolism.

Trial registration: Clinicaltrials.gov ID no.: 00918918

Introduction

It is recognized that chronic, low-grade inflammation is an important hall mark in the development of both atherosclerosis and type 2 diabetes (1;2). Low-grade inflammatory changes have been shown to precede type 2 diabetes by many years (3;4). Moderate alcohol consumption has been consistently associated with a reduced risk of both cardiovascular diseases (5) and type 2 diabetes (6) compared with abstention possibly due to a lowered inflammatory status.

Alcohol consumption is known to modulate several immune functions and inflammatory processes (7). In observational studies moderate alcohol consumption is associated with lower levels of inflammatory markers (8) and lowered levels of C-reactive protein (CRP) (9-11). Data from randomized trials on inflammatory markers after prolonged moderate alcohol consumption are scarce but do suggest lower levels of certain cytokines (12) and CRP (13;14) although not consistently (15). Acute moderate alcohol consumption has been shown to suppress the production of pluripotent nuclear transcription factor kappa B (NF- κ B) (16-18) in white blood cells. Activation of NF- κ B, induced by a wide range of signals including lipopoly-saccharides and viruses, is a pivotal step in the induction of several inflammatory cytokines, chemokines and growth factors.

Besides their important role in inflammatory responses and the immune system, white blood cells are also the most readily available tissue for gene expression profiling. More importantly, their gene expression profiles have previously been shown to reflect dietary-induced changes in several short-term and longer-term intervention trials (19;20).

Recently, we have shown that the interpretation of a dietary intervention-induced changes can be strongly facilitated by the use of an integrative approach of comprehensive techniques such protein and gene profiling (21). Hence, we examined the effects of prolonged moderate alcohol consumption by means of an integrated approach of gene and protein profiling of blood.

Methods

Study Design

The study used a randomized, open-label, crossover design, consisting of two 4-week periods. Subjects consumed two miniatures (50 mL each) of vodka (Smirnoff, U.K.) (30 g alcohol) with 200 mL orange juice (Appelsientje, the Netherlands) or only orange juice daily for four weeks during dinner. The study was conducted at TNO (a Dutch acronym for applied scientific research) Quality of Life, in Zeist, the Netherlands. The study was approved by an independent centralized ethics committee (METOPP; Tilburg, the Netherlands) and is registered at Clinicaltrials.gov, number NCT 00918918. Allocation to treatment order was randomized according to BMI and age. Blood sampling was done on the morning after four weeks of each treatment period, after an overnight fast. Compliance was monitored by weekly measurement of urinary ethyl glucuronide (EtG), a direct phase II metabolite of ethanol formed by action of UDP-glucuronosyl transferase. EtG has been

reported to be a superior marker with 100% sensitivity as a biomarker of recent drinking. It persists in the urine up to ~75 - 85 h after last intake (22). Cut-off limit to assess compliance during juice intervention for urinary EtG values was set at >0.25 mg/mL (positive sample), to obtain a high sensitivity but avoid positive results due to unintentional ethanol exposure. Cut-off limit during vodka intervention was set at EtG values <0.5 mg/mL (2.2 μ mol/L) (negative sample). Additional measures of compliance were the increase of HDL-cholesterol and the return of empty bottles of vodka.

Subjects

We recruited 24 apparently healthy, non smoking male subjects between 21 and 40 years. Subjects were eligible if they consumed between 5 and 28 units of alcohol per week, were free of any medication at the beginning of the study, and had a BMI between 18 and 27 kg/m² and had no family history of alcoholism. Subjects gave written informed consent and received a compensation for their participation. They were instructed to maintain their habitual body weight, food pattern and physical activity pattern and were told to refrain from any alcoholic products during the entire study except the vodka supplied by TNO.

Blood and urine handling and analysis

Blood samples were obtained from the antecubital vein of the forearm and collected in tubes containing clot activator for serum and in ice-chilled tubes containing or Potassium Ethylene Diamine Tetra Acid (K₃EDTA) for plasma (Vacutainer Systems, Becton Dickinson, Plymouth, U.K.). Blood was centrifuged for 15 minutes at 2.000 g at 4°C, within 15 - 30 minutes after collection and stored at <-70°C. Serum clinical chemistry tests included the measurement of γ -glutamyltransferase, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, glucose, insulin, triglycerides, free fatty acids total cholesterol, HDL-cholesterol, LDL-cholesterol, and phospholipids were analyzed with enzymatic techniques (Boehringer-Mannheim, Mannheim, Germany) on an Olympus AU400 clinical chemistry analyzer (Olympus-Diagnostica Europe, Hamburg, Germany). Six serum apolipoproteins (apolipoproteins A-I, A-II, B, C-I, C-III and E) and 47 plasma proteins (Human InflammationMAP™ 1.0; <http://www.rulesbasedmedicine.com/products-services/Human-InflammationMAP.asp>) were analyzed by bead-based multiplexed immunoassay at the biomarker testing laboratory Rules Based Medicine (RBM; Austin, Texas, U.S.A.). Total adiponectin was measured using MesoScale Diagnostics assays (Cat# K151IYC-1) (Meso Scale Discovery; Gaithersburg, MD, U.S.A.). For gene expression analysis 2.5 mL blood was collected in PAXgene tubes (BD, Erembodegem, Belgium) and stored according to the manufacturer's instructions. EtG was determined in morning urine samples as previously described (23).

RNA extraction and handling

RNA was isolated using PAXgene Blood RNA Kit (Qiagen, Venlo, the Netherlands) according to manufacturer's instructions. The Quality control, RNA labeling, hybridization and data extraction were performed at ServiceXS (Leiden, the Netherlands). RNA concentration was measured using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, U.S.A.). The RNA quality and integrity was determined using Lab-on-Chip analysis on an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, U.S.A.). Average RNA yield of the samples was 10.3 μg (range 1.9 - 28 μg) and the average RNA Integrity Number (RIN) score was 8.9 (range 6.8 - 9.6). The labeling reaction included a globin reduction step. Four different peptide nucleic acids (PNAs) were used to block the 3' ends of globin transcripts. Due to the addition of the globin PNA sequences to the labeling reaction, the labeling of globin transcripts is hampered in favor of the other transcripts, which are typically underrepresented in blood cells. As a consequence, the signal strength of non-globin genes is much higher after globin reduction. Biotinylated cRNA was prepared using the Illumina TotalPrep RNA Amplification Kit (Ambion, Inc., Austin, TX, U.S.A.) according to the manufacturer's specifications starting with 500 ng total RNA. Per sample, 750 ng of cRNA was used to hybridise to the Sentrix HumanHT-12 v3 Expression BeadChips (Illumina, Inc., San Diego, CA, U.S.A.). Each BeadChip contains twelve arrays. Hybridization and washing were performed according to the Illumina standard assay procedure. Scanning was performed on the Illumina iScanner (Illumina, Inc., San Diego, CA, U.S.A.). Image analysis and extraction of raw expression data was performed with Illumina Genomestudio 2010 Gene Expression software with background subtraction and no normalization. Probe level data were submitted to quantile normalization in GeneSpring GX 11.0 (Agilent Technologies, Santa Clara, CA, U.S.A.) and probes were filtered on detection P value (>0.99 in at least one sample). This resulted in normalized data for 19,378 probes.

Statistical analysis

Serum and plasma variables at the end of the intervention were compared between treatments with a mixed analysis of variance model that included terms for treatment, period and the interaction between period and treatment (indicating possible carryover effects). The so-called 80% rule was applied to the multiplex inflammatory protein dataset to retain only those plasma analytes for which $\geq 80\%$ of the values were above the detection limit for ≥ 1 of the 2 treatment groups (24), resulting in retention of 29 of the 47 antigens measured in plasma. Values below the detection limit that remained in the truncated data set were replaced by a value of half of the detection limit. Values for remaining samples that were not measurable on the standard curve for a specific protein were set at 0.1 times the detection limit for that protein. Statistical analyses were performed using the SAS statistical software package (SAS version 8.2 and 9, SAS Institute, Cary, NC, U.S.A.). Statistical significance was defined as $P < 0.05$. For gene expression data, an additional threshold was applied to select genes that were up-regulated ($>0\%$) in ≥ 15 subjects or down-regulated ($<0\%$) in ≥ 15 subjects (Figure 5.1). The cut-off of 15 subjects was based on

the average number of subjects that showed intervention-induced alterations in significantly changed circulating markers. These remaining genes were used for biological interpretation and network construction.

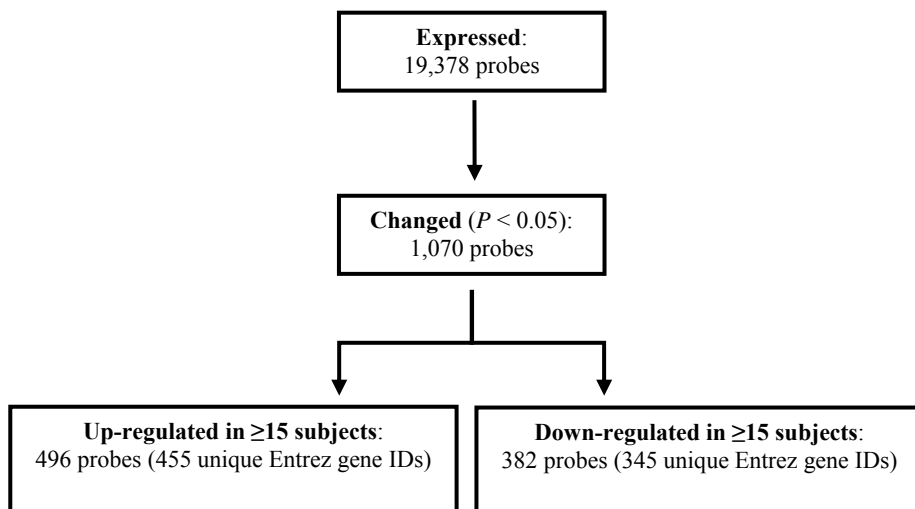


Figure 5.1: Flow chart of probe selection and number of probes and specific genes changed between the two interventions in the microarray analysis.

Biological interpretation

Functional analysis of the gene expression data was performed by using Ingenuity Pathway Analysis version 8.7 (Ingenuity Systems Inc, Redwood City, CA, U.S.A.), identifying significantly enriched pathways and gene lists ($P < 0.05$). Networks were built in MetaCore version 6.1 (GeneGo, St Joseph, MI, U.S.A.), using direct interactions or shortest path algorithm (max 2 steps and with filter for human data).

Results

Subjects and compliance

All 24 men enrolled completed the study without any serious adverse events. Mean (\pm SEM) age and BMI of the men were 25.5 ± 3.3 y and 23.5 ± 2.5 kg/m². Weekly urinary EtG levels were assessed to check individual compliance to treatments. This revealed eight positive urine samples during the orange juice-drinking period, although all ≤ 0.5 mg/mL, and seven negative samples during the vodka orange juice-drinking period out of the 192 samples analyzed (overall compliance of 92%). Another indicator for compliance was the 8.8% increase in HDL-cholesterol after vodka and juice compared with juice consumption (Table 5.1).

Table 5.1: Overview of circulating proteins and metabolites that significantly differed ($P < 0.05$) between the two 4-week treatments in 24 young men and sampled after an overnight fast.

	Orange Juice	Vodka and orange juice	% change	P value ¹
Lipoproteins				
HDL-cholesterol (mmol/L)	1.12 ± 0.04	1.22 ± 0.05	8.8	< 0.001
Phospholipids (mmol/L)	2.36 ± 0.08	2.52 ± 0.08	6.0	< 0.001
Apolipoprotein A-II (ng/L)	221 ± 13	243 ± 13	11.6	< 0.01
Apolipoprotein C-I (ng/L)	239 ± 8	255 ± 8	7.7	0.01
Apolipoprotein C-III (µg/L)	84 ± 6	94 ± 6	10.4	0.02
Inflammatory markers				
Total adiponectin (µg/mL)	10.6 ± 0.9	11.6 ± 0.9	8.6	< 0.01
IL-1 receptor agonist (pg/mL)	68 ± 5	52 ± 5	-13.8	< 0.01
IL-18 (pg/mL)	152 ± 13	143 ± 13	-4.0	0.03
Miscellaneous/liver markers				
γ-glutamyltransferase (U/L)	19.8 ± 2.4	24.3 ± 2.4	18.7	< 0.001
Ferritin (ng/mL)	125 ± 18	100 ± 18	-13.6	< 0.01
Alpha-1 antitrypsin (mg/mL)	1.49 ± 0.05	1.39 ± 0.05	-6.0	0.03

Data are expressed means (± SEM).

¹ P values obtained from a mixed ANOVA model that included terms for treatment, period and the interaction between period and treatment (to assess carryover effects).

Serum and plasma measurements

No significant ($P > 0.05$) carryover effects as assessed by an interaction between treatment and period effect were observed for any plasma or serum measures. As expected HDL-cholesterol and some HDL-associated apolipoproteins such as apolipoprotein (apo)A-II and apoC-I, apoC-III and phospholipids increased (all $P < 0.05$) after alcohol consumption (Table 5.1). Plasma levels of the pro-inflammatory cytokines interleukin (IL)-1 receptor antagonist and IL-18 were significantly reduced (both $P < 0.05$) whereas plasma levels of the anti-inflammatory protein adiponectin were increased after vodka consumption ($P < 0.05$).

Table 5.2: Overview of differentially expressed pathways and number of genes changed based on Ingenuity Pathway Analysis, ranked on most significantly changed pathway in functional group.

Functional group	Pathways / lists	P value	# changed
Signaling	Glucocorticoid receptor signaling	0.0017	22
	Acute myeloid leukemia signaling	0.0047	9
	Estrogen receptor signaling	0.0151	10
	Chronic myeloid leukemia signaling	0.0158	9
	PI3K/AKT signaling	0.0204	10
	Neuregulin signaling	0.0288	8
	BMP signaling pathway	0.0324	7
	GNRH signaling	0.0347	10
	G Beta gamma signaling	0.0372	8
	Androgen signaling	0.0417	9
Immune response	Antigen presentation pathway	0.0028	7
	Crosstalk between dendritic cells and natural killer cells	0.0044	10
	Leukocyte extravasation signaling	0.0095	14
	Oncostatin M signaling	0.0135	5
	PKC θ , signaling in T lymphocytes	0.0151	10
	GM-CSF signaling	0.0155	7
	B Cell receptor signaling	0.0166	12
	IL-15 signaling	0.0407	6
	T Cell receptor signaling	0.0457	8
	Role of NFAT in regulation of the immune response	0.0468	12
(Lipid) metabolism	Mechanism of gene regulation by peroxisome proliferators via PPAR α	0.0065	10
	Citrate cycle	0.0069	5
	PPAR α /RXR activation	0.0257	12
Other	Role of NFAT in cardiac hypertrophy	0.0178	14
	Selenoamino acid metabolism	0.0214	5
Apoptosis	Apoptosis signaling	0.0224	8
	Pro-apoptosis	0.0316	5
	TNF-R1 signaling	0.0479	5

Microarray analysis and pathway analysis

Microarray hybridization was performed on RNA extracted from whole blood which was collected at the end of each 4-week intervention period. All 48 arrays passed the quality-control criteria. Of the 48,803 probes present on the microarray, 19,378 probes were defined as expressed in the white blood cells (Figure 5.1). Four weeks of vodka and juice resulted in down-regulation of 382 probes (345 genes with unique Entrez IDs) and up-regulation of 492 probes (455 genes with unique Entrez IDs) in leukocytes.

To determine the role of genes that changed after vodka and juice consumption, we performed pathway analysis in Ingenuity Pathway Analysis. This revealed changes in pathways related to diverse signaling processes, immune response, lipid metabolism/PPAR α and apoptosis (Table 5.2).

The functional group ‘signaling’ contained a diversity of the different signaling processes. Two of the top four pathways of this functional group involved pathways related to myeloid leukemia signaling. Table 5.3 shows the genes involved in these pathways of which expression levels were significantly different between the treatments.

Another functional group that was altered by the alcohol treatment compared to the alcohol free treatment was ‘immune response’ and contained among others the pathway of antigen presentation, B and T cell receptor signaling and IL-15 signaling. Forty unique genes were involved in this functional group. These genes were, together with the significantly affected proteins related to immune response/inflammation (e.g. total adiponectin, IL-1 receptor antagonist, IL-18, ferritin, and alpha-1 antitrypsin) used for network analysis in MetaCore (Figure 5.2). This integrative network analysis of genes and proteins revealed coherent effects of moderate alcohol consumption on immune response, as indicated by the direct links in the network in Figure 5.2. The figure depicted a central role for the transcription factor NF- κ B.

Similarly, a network was created for genes from the pathway category (lipid) metabolism together with HDL-cholesterol and the significantly changed HDL-associated markers (e.g. apolipoproteins and total adiponectin). Again, this network approach illustrated close biological links between the alcohol-induced changes in genes and proteins involved with a central role for PPAR α (Figure 5.3).

Table 5.3: Genes involved in myeloid leukemia signaling (acute and chronic) which expression levels differed between treatments[†].

Gene description	Gene name	Entrez ID	Mean 2 log ratio	P value
Histone deacetylase 5	<i>HDAC5</i>	10014	-0.52	0.019
Runt-related transcription factor 1	<i>RUNX1</i>	861	-0.47	0.020
Growth factor receptor-bound protein 2	<i>GRB2</i>	2885	-0.26	0.030
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2	<i>NFKB2</i>	4791	-0.18	0.043
Signal transducer and activator of transcription 5A	<i>STAT5A</i>	6776	-0.16	0.011
Proliferation-associated 2G4	<i>PA2G4</i>	5036	-0.15	0.026
CCAAT/enhancer binding protein	<i>CEBPA</i>	1050	-0.13	0.021
V-raf-1 murine leukemia viral oncogene homolog 1	<i>RAF1</i>	5894	0.12	0.049
V-crk sarcoma virus CT10 oncogene homolog	<i>CRK</i>	1398	0.13	0.047
V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	<i>KRAS</i>	3845	0.17	0.033
Colony stimulating factor 2 receptor	<i>CSF2RB</i>	1439	0.57	0.016
V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	<i>KIT</i>	3815	0.60	0.007

[†] $P < 0.05$ and an additional restriction of up- or down-regulation of the gene expression level in at least 15 of the 24 subjects.

Discussion

In this study, we assessed changes in gene expression and circulating markers after moderate alcohol consumption in a randomized crossover trial in men. With an integrated approach of gene and protein profiling of blood, we showed that four weeks of moderate alcohol consumption (30 g alcohol per day) resulted in changes related to immune response and lipid metabolism. The altered gene expression profiles in immune response were predominantly associated with a decreased NF- κ B expression whereas changes in lipid metabolism were in close connection with PPAR α .

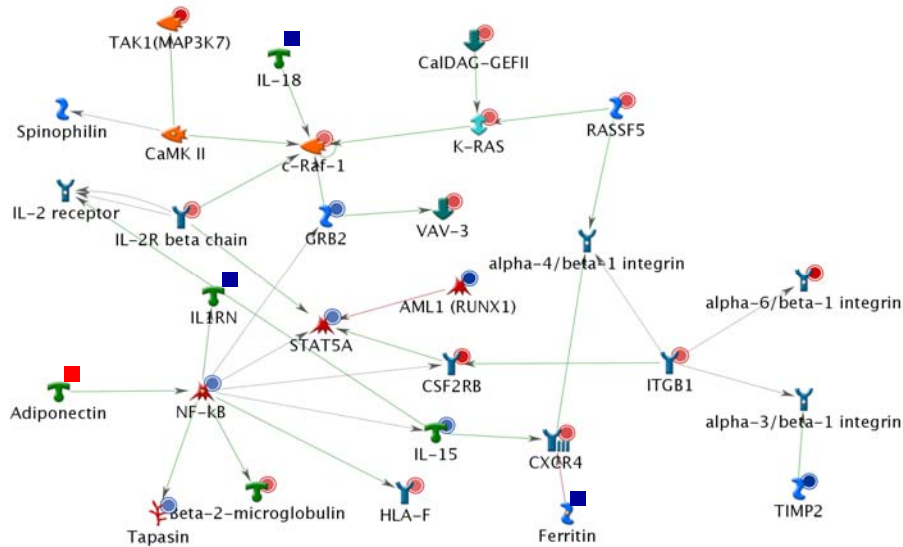
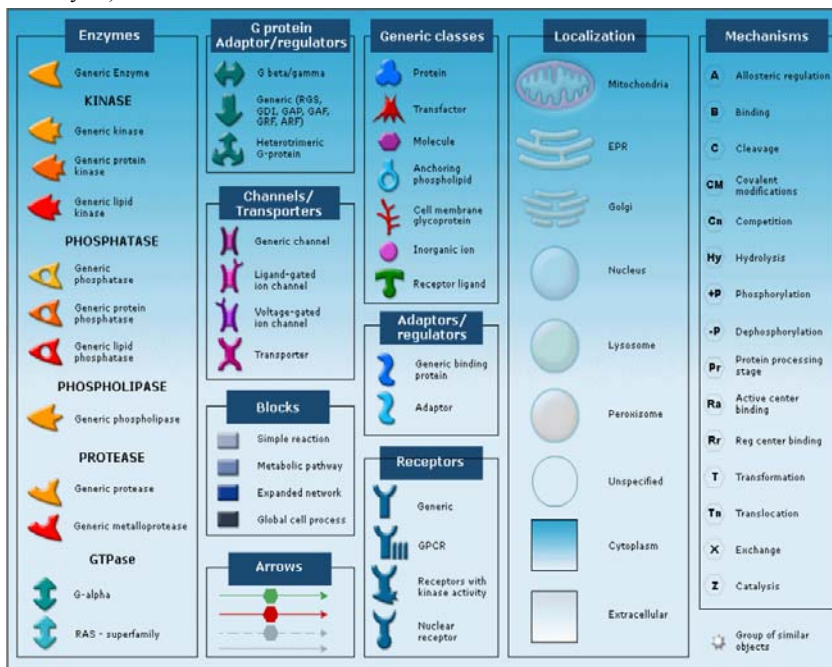


Figure 5.2: Immune response network with a central role of nuclear transcription factor kappa B (NF-κB). The network was derived using MetaCore and based on direct interactions between 40 genes involved in immune response and 5 plasma proteins. Blue circles/squares indicate down-regulation in response to alcohol intervention, red circles/squares indicates up-regulation in response to alcohol intervention. Squares denote proteins (measured in plasma), circles represent genes (measured in leukocytes).



We observed altered expression profiles in white blood cells related to acute and chronic myeloma and lymphoma signaling. Expression of the genes encoding for signal transducer and activator of transcription 5A (*STAT5A*) and runt-related transcription factor 1 (*RUNX1*) (a.k.a. *AML1*, acute myeloid leukemia 1) were down-regulated after moderate alcohol consumption compared with the abstention period. Activation of *STAT5A* has been shown to be essential for the tumorigenesis. The altered expression levels in pathways of myeloma and lymphoma signaling in our crossover study might provide mechanistic support for findings of several observational studies (25;26). In these large prospective cohort studies an inverse association between alcohol consumption and risk of non-Hodgkin lymphoma was reported.

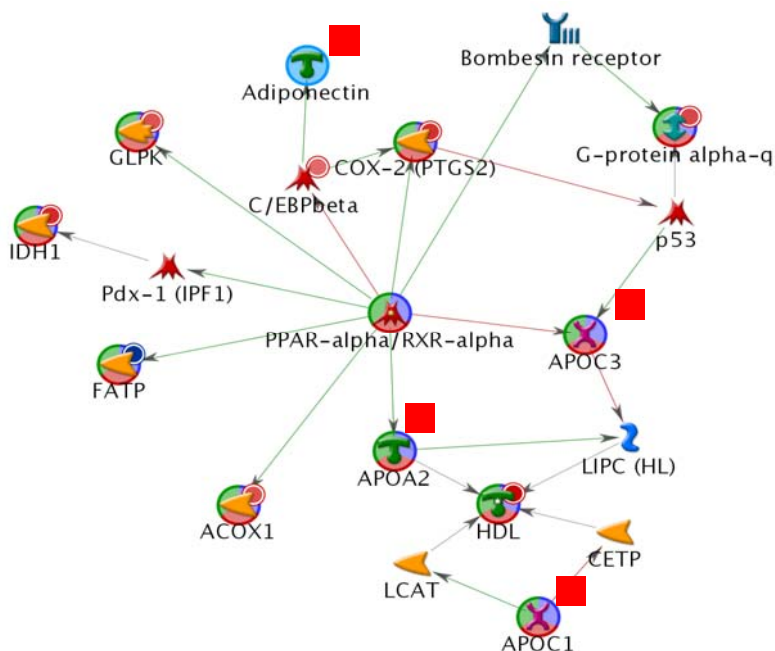


Figure 5.3: Network around peroxisome proliferator-activated receptor alpha (PPAR α).

The network was derived using MetaCore and based on shortest path (max 2 steps) between 11 genes involved in PPAR regulated pathways or citrate cycle and 4 plasma proteins. Blue circles/squares indicate down-regulation in response to the alcohol intervention, red circles/squares indicate up-regulation in response to alcohol intervention. Squares denote proteins (measured in plasma), circles represent genes (measured in leukocytes).

We observed a down-regulation of NF- κ B after prolonged moderate vodka and juice consumption compared to juice only consumption. The various direct connections of NF- κ B with other immune-related gene expression and protein levels underlined the pleiotropic regulator function of NF- κ B in inflammatory responses. Our findings of prolonged alcohol consumption on NF- κ B are in concord with observations after acute consumption in which is shown that acute alcohol prevents NF- κ B activation which lead to an inhibition in pro-inflammatory cytokines such as TNF- α and IL-1 β at the protein as well as the mRNA level (18). We did observe lower IL-18 levels after moderate alcohol consumption, in line with a previous study among diabetic men (27).

We and others have previously reported increased circulating adiponectin concentrations after moderate alcohol consumption (28-30). Adiponectin was present in both the immune response and PPAR α /lipid metabolism networks. This adds to the notion that adiponectin might be an important link between inflammation and lipid metabolism. Indeed several lines of evidence suggest an inverse association between adiponectin and markers of inflammation (31) and with favorable lipid profiles (32) in observational studies. Gene expression of *ADIPOQ* in these white blood cells did not change between treatments, despite the increased levels after moderate alcohol consumption. However, adiponectin is predominantly expressed in the adipose tissue. The alcohol-induced increase of the protein can largely be attributed to increased expression of the adiponectin gene in adipose tissue (23).

We found a substantial decrease in plasma ferritin concentrations after the vodka treatment compared with the control treatment. Ferritin is one of the key proteins regulating iron homeostasis. Growing evidence has shown that even moderately increased iron stores, represented by high-normal ferritin concentrations, are associated with insulin resistance (33) and type 2 diabetes. The results of our randomized trial are in contrast with observational research which shows a positive association with alcohol consumption and ferritin concentrations (34-36). If confirmed in other trials, decreased ferritin concentrations might be a new and relatively unexplored mechanism that may explain the lowered risk of type 2 diabetes associated with moderate consumption. Gene expression of ferritin in white blood cells did not change between treatments, probably because the protein is predominantly expressed in liver tissue.

The observations of increased HDL-cholesterol and apolipoproteins after alcohol consumption are consistent with previous findings in 132 healthy men (37). Previous studies by our group showed similar increases of HDL-cholesterol in young men (38) and middle-aged men (39;40) and women (41) after alcohol consumption.

Strong points of the study are its randomized crossover design, the high compliance to the treatments throughout the study and the integrated approach of both proteins and gene expression profiles. However, several limitations warrant consideration. First, we used a specific type of tissue, i.e. white blood cells, for gene profiling. Although these cells might not be representative for all tissues directly affected by alcohol consumption (e.g. liver and adipose tissue), these cells were chosen because of their

important roles in inflammatory responses and the immune system. Furthermore, their expression profiles have previously been shown to reflect metabolic changes due to short-term and longer term nutritional adaption in healthy humans (20;21). Second, whole blood was collected for RNA isolation. As globin RNA was removed from the samples the gene expression profiles can be assigned to the white blood cell population. Since the white blood cell population consists of a range of cell types present in the blood samples, it can not be completely excluded that changes in gene expression might reflect changes in white blood cell type distribution in blood. Third, we only measured specific proteins based on *a priori* expected changes (inflammatory markers and lipoproteins) which might have biased our results. However, the analysis of whole-genome expression data and subsequent pathway analyses were relatively unbiased, since all genes were measured and this analysis indicated the same processes being affected. Furthermore, the network analysis revealed integrated effects (shown by the direct connections in both immune response and lipid/lipoprotein metabolism network) which might represent a close biological connection between the changes in genes and proteins due to alcohol consumption.

In conclusion, a comprehensive and integrated approach of gene and protein profiling of blood showed that moderate alcohol consumption resulted in changes in immune response and lipid metabolism. Our integrated and large-scale profiling revealed potential new functional pathways and physiological mechanisms that may explain some of the health effects that moderate alcohol consumption exerts.

Authors' contributions

MMJ provided partial funding, designed the study, analyzed and interpreted the data and wrote the manuscript. MJE and LP participated in data collection, analyzed and interpreted the data and helped in drafting the manuscript. RFW helped designing the study and critically reviewed the content for important intellectual content. HFJH provided funding, designed the study and critically reviewed the manuscript for important intellectual content. All authors read and approved the final manuscript.

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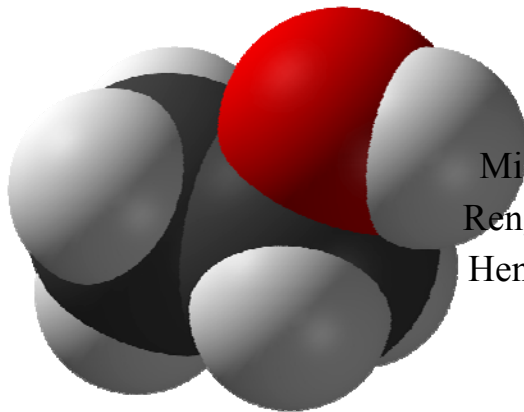
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Chapter 6

Alterations in total and high-molecular weight adiponectin after three weeks of moderate alcohol consumption in premenopausal women



Michel M. Joosten
Renger F. Witkamp
Henk F.J. Hendriks

Submitted in revised form

Background: Moderate alcohol consumption is associated with a reduced risk of type 2 diabetes. It also increases concentrations of adiponectin, a hormone inversely associated with type 2 diabetes risk. However, the rate at which this increase occurs is unclear.

Objective: To examine the effect of moderate alcohol consumption on the weekly alterations in levels of both total and high-molecular weight (HMW) adiponectin and associated markers of glucose and lipid metabolism.

Methods: In a randomized, open-label, crossover trial, 24 normal-weight, premenopausal women who were regular alcohol consumers, received 66 cL of beer (~26 g alcohol) or 66 cL of alcohol-free beer (<0.5 g alcohol) daily for 3 weeks preceded by a 1-week washout. Blood samples were collected weekly after an overnight fast for measurement of plasma total and HMW adiponectin and markers of glycemia and lipid metabolism.

Results: There was a significant interaction ($P < 0.05$) between the two treatments over time for both plasma HWM and total adiponectin concentrations. Within three weeks, moderate alcohol consumption significantly increased plasma total (8.2%; $P = 0.01$) and HMW (8.2%; $P = 0.02$) adiponectin levels compared with abstention. Changes over time in total adiponectin were positively associated with changes in HMW adiponectin during the nonalcoholic beer ($r = 0.80$; 95% confidence interval (CI): 0.55, 0.92) and beer ($r = 0.82$; 95% CI: 0.58, 0.93) intervention. Alcohol consumption did not affect the ratio HMW to total adiponectin nor serum glucose, insulin, hemoglobin A_{1c} or triglyceride levels compared with abstention during the 3-week intervention periods.

Conclusions: Moderate alcohol consumption concomitantly increases plasma total and HMW adiponectin concentrations compared to abstention in premenopausal women. These effects were evident after at least three weeks of consumption. No changes were observed in other markers of insulin sensitivity.

Trial registration: Clinicaltrials.gov ID no.: NCT00524550.

Introduction

Moderate alcohol consumption is associated with a reduced risk of type 2 diabetes (1;2). This association could – in addition to improved insulin sensitivity and favorable changes in triglycerides (3;4) – partially be explained by increased circulating adiponectin levels (5). Adiponectin is an adipose tissue-specific adipokine involved in glucose and lipid metabolism (6). It is positively associated with insulin sensitivity (7) and inversely with inflammatory markers (8). Moreover, higher adiponectin levels have been consistently associated with a lower risk of type 2 diabetes (9) and cardiovascular disease (10).

In observational studies alcohol consumption has been positively associated with adiponectin concentrations (11-13), an effect confirmed in short-term randomized trials of alcohol administration (14-17).

Acute alcohol consumption, however, does not alter postprandial levels of adiponectin (18;19) suggesting a longer period of alcohol consumption is needed to increase adiponectin levels. Also, almost all of these short-term trials only included men whereas adiponectin levels differ between men and women (20) and lower levels are observed in premenopausal compared to postmenopausal women (21;22). Moreover, the majority of these trials investigated only total adiponectin levels, whereas the high-molecular weight (HMW) isomer of adiponectin has been proposed as the most active metabolic form (23-25).

Hence, this trial was designed to investigate the weekly alterations in levels of both HMW and total adiponectin and associated markers of glucose and lipid metabolism by changes in alcohol consumption over a 3-week period in a group of premenopausal women.

Methods

Study design

The study used a randomized, open label, placebo-controlled, crossover design, consisting of two 3-week periods, each preceded by a one week washout. Subjects daily consumed two cans (66 cL) of beer (~26 g alcohol) or two cans of alcohol-free beer (Amstel, the Netherlands) for three weeks during dinner. The study was conducted at TNO (a Dutch acronym for applied scientific knowledge) Quality of Life (Zeist, the Netherlands). Subjects were instructed to maintain their habitual body weight, food and physical activity pattern and were told not to consume any additional alcohol during the entire study (including washout periods). Body weight was measured weekly wearing indoor clothing, without shoes, wallet and keys using a digital weight scale with a precision of 0.1 kg (SECA, model 701, Germany). Blood and urine sampling was done weekly for eight weeks after an overnight fast. Compliance was monitored by weekly measurement of ethyl glucuronide, a direct phase II metabolite of alcohol consumption in the urine and increase in serum HDL-cholesterol. Cut-off limit to assess compliance during alcohol free intervention for urinary ethyl glucuronide values was set at >0.25 mg/mL (positive sample), to obtain a high

sensitivity but avoid positive results due to unintentional ethanol exposure. Cut-off limit during the beer intervention was set at ethyl glucuronide values <0.5 mg/mL (2.2 μ mol/L) (negative sample) (4).

Study subjects

Subjects were recruited from a pool of volunteers of TNO Quality of Life. Eligible premenopausal women consumed between 5 and 21 units of alcohol per week, were apparently healthy, between 20 to 40 years, used phase I or II oral contraceptives, had a BMI between 19 and 25 kg/m², refrained from smoking and had no family history of alcoholism. They gave written informed consent and received compensation for their participation. The study was approved by an independent centralized ethics committee (METOPP, Tilburg, the Netherlands). This trial is registered at Clinicaltrials.gov, number NCT00524550.

Handling and analysis of blood and urine samples

Ethyl glucuronide samples in morning urine were sampled and treated as described previously (4). Blood samples were obtained weekly from the antecubital vein of the forearm and collected in tubes containing clot activator for serum and in ice-chilled tubes containing or potassium ethylene diamine tetra acid (K₃EDTA) for plasma (Vacutainer Systems, Becton Dickinson, Plymouth, U.K.) and were centrifuged for 15 minutes at 2.000 g at 4°C, within 15 - 30 minutes after collection and stored at $<-70^{\circ}\text{C}$. Biochemical determinations in blood were performed at TNO Quality of Life using Olympus analytical equipment and reagents except for adiponectin. Plasma total and HMW adiponectin concentrations were determined by an enzyme-linked immunosorbent assay (ELISA) (Catalogue number: 47-ADPHU-E01; Alpco Diagnostics, Salem, NH, U.S.A.). The intra-assay coefficients of variation for the two forms of adiponectin were 5.4% for total and 5.0% for HWM adiponectin.

Statistics

Variables over time were compared between treatments using a mixed analysis of variance model that included fixed terms for treatment, time, treatment sequence (indicating possible carry-over effects), and the interaction between treatment and time and random terms for subject and period. Body weight was included in the model as a random factor to correct for potential fluctuations in body weight. Orthogonal polynomials were used to test for linear or quadratic trends. Regression analyses were performed to describe the slopes of the time trend curve for both beverages. For the correlation between changes over time in adiponectin and HMW adiponectin, a Fisher's z transformation was applied on individual correlations to correct for deviations from the normal distribution and a 95% confidence intervals (CI) were calculated. Statistical analyses were performed using the SAS statistical software package (SAS version 8, SAS Institute, Cary, NC, U.S.A.). Data are presented as mean \pm SEM unless specified otherwise. Statistical significance was defined as $P < 0.05$.

Results

All 24 enrolled women completed the study (Table 6.1). Mean age and BMI of the women were 23.9 ± 4.3 y and 22.2 ± 1.6 kg/m² respectively. No carryover effects were seen in any outcome measure. Analysis of urinary ethyl glucuronide revealed two negative samples during the beer-intervention and no positive samples during the alcohol free beer intervention or washout period out of the 191 samples analyzed (overall compliance of 99.0%). Another measure of compliance was the increase ($P < 0.01$) in HDL-cholesterol after three weeks of beer compared with non-alcoholic beer consumption (Table 6.2).

Table 6.1: Baseline characteristics of twenty-four premenopausal women after an overnight fast.

Variable	Mean \pm SD	Range
Body weight (kg)	67.6 ± 6.5	54.4 - 79.4
Glucose (mmol/L)	4.95 ± 0.28	4.39 - 5.39
Insulin (pmol/L)	41.7 ± 16.0	20.1 - 81.3
HDL-cholesterol (mmol/L)	1.61 ± 0.33	1.06 - 2.17
LDL-cholesterol (mmol/L)	2.62 ± 0.46	1.70 - 3.40
Triglycerides (mmol/L)	1.11 ± 0.40	0.42 - 1.98
Alanine aminotransferase (units/L)	16.9 ± 6.4	9 - 30
Aspartate aminotransferase (units/L)	21.0 ± 5.7	13 - 41
Alkaline phosphatase (units/L)	62.6 ± 15.6	33 - 86
γ -Glutamyltranspeptidase (units/L)	19.0 ± 9.1	7.6 - 39.2

Mean body weight was slightly higher during the 3-week beer drinking period (67.9 ± 1.3 kg vs. 67.6 ± 1.3 kg, $P < 0.01$). Results were adjusted for this difference in body weight, but did not essentially change. Therefore, unadjusted results are presented. Plasma levels of total and HMW adiponectin over time differed between the two treatments ($P_{\text{interaction}} < 0.05$; Figure 6.1). After the first two weeks, adiponectin levels did not differ between treatments. However, after three weeks, both HMW (3.73 ± 0.36 μ g/mL vs. 3.47 ± 0.36 μ g/mL, $P = 0.02$) and total (7.24 ± 0.51 μ g/mL vs. 6.77 ± 0.51 μ g/mL, $P = 0.01$) adiponectin levels were 8.2% higher after consuming beer compared with consuming non-alcoholic beer. No differences between treatments over time or between treatments were observed in the ratio HMW to total adiponectin or levels of serum glucose, insulin, triglycerides or free fatty acids (data not shown).

Table 6.2: Characteristics of twenty-four premenopausal women after three weeks of consuming beer or alcohol-free beer after an overnight fast.

	Alcohol-free	Beer	<i>P</i> value [†]
Glycemic markers			
Glucose (mmol/L)	4.79 ± 0.11	4.84 ± 0.11	0.36
Insulin (pmol/L)	45.7 ± 4.0	46.1 ± 4.0	0.90
Hemoglobin A _{1c} (%)	5.0 ± 0.04	4.9 ± 0.04	0.16
Lipid profile			
HDL-cholesterol (mmol/L)	1.52 ± 0.07	1.62 ± 0.07	< 0.01
LDL-cholesterol (mmol/L)	2.40 ± 0.07	2.37 ± 0.07	0.77
Triglycerides (mmol/L)	1.27 ± 0.08	1.25 ± 0.08	0.61
Free fatty acids (mmol/L)	0.34 ± 0.03	0.29 ± 0.03	0.26
Liver enzymes			
Alanine aminotransferase (units/L)	10.8 ± 1.8	10.0 ± 1.8	0.21
Aspartate aminotransferase (units/L)	17.8 ± 2.0	17.8 ± 2.0	0.95
Alkaline phosphatase (units/L)	56.9 ± 6.5	57.8 ± 6.5	0.68
γ-Glutamyltranspeptidase (units/L)	16.5 ± 2.2	18.5 ± 2.2	0.01

Data are expressed as mean ± SEM.

[†] Mixed ANOVA model on baseline values that included terms for treatment, period and the interaction between period and treatment (to assess carryover effects).

The orthogonal polynomial describing the linear time trend for each adiponectin form was significantly different between the two interventions ($P_{\text{interaction}} < 0.01$). During the 3-week abstinence period, the slope for HMW was $-0.27 \pm 0.09 \mu\text{g/mL}$ ($P < 0.01$) and for total adiponectin levels $-0.54 \pm 0.13 \mu\text{g/mL}$ ($P < 0.001$) whereas the slopes of the two adiponectin forms during the alcohol period did not differ from zero. Changes over the weeks in total adiponectin were positively associated with changes in HMW adiponectin during the nonalcoholic beer ($r = 0.80$; 95% CI: 0.55, 0.92) and beer ($r = 0.82$; 95% CI: 0.58, 0.93) intervention. Changes over time in the two adiponectin forms were not associated with changes in serum glucose, insulin, triglycerides and free fatty acids.

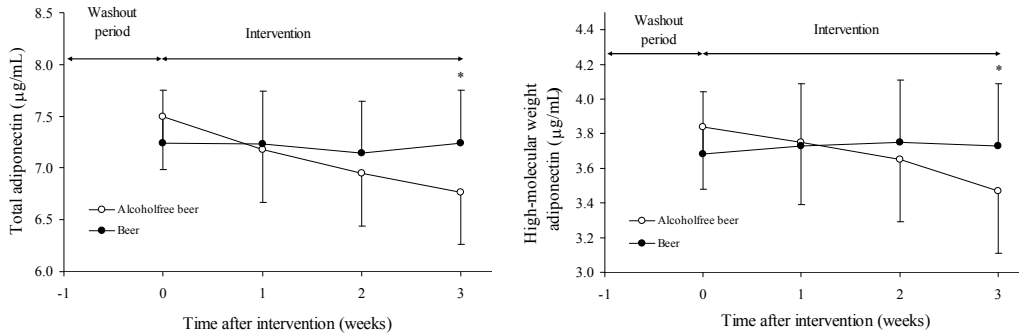


Figure 6.1. Mean \pm SEM weekly plasma concentrations of total adiponectin and high-molecular weight adiponectin during a 3-week intervention with daily beer (●) or alcohol-free beer consumption (○) each preceded by a 1-week washout among twenty-four normal-weight premenopausal women.

* $P < 0.05$ between beer and alcohol-free beer consumption at time-point during the intervention.

Discussion

The present study showed that among young, normal-weight women moderate alcohol consumption, consumed daily with dinner, increased both HMW and total adiponectin concentrations compared with abstinence. These increases were evident after at least three weeks of consumption. Furthermore, the changes over time in HMW and total adiponectin levels during each treatment were correlated, which suggest that these changes occurred concomitantly. No changes were observed in markers of glycemia or lipid metabolism in these women.

A trial by Imhof and colleagues (14) investigated the effects on total adiponectin levels after three weeks of daily alcohol consumption among women (20 g alcohol/day) and men (30 g/day). They reported increased adiponectin levels after red wine but not after beer consumption in women and higher levels after beer and ethanol but not after red wine consumption in men. The authors hypothesized that drinking preferences of participants might substantially have affected the findings by incomplete adherence to the study protocol as women preferred to drink wine whereas men preferred to drink beer. Other experimental studies have consistently reported similar robust increases in circulating total adiponectin levels in young (17) and middle-aged men (15;16) and postmenopausal women (4), irrespective of beverage type. Moreover, we have previously reported that alcohol intervention-associated changes in mRNA levels of the gene encoding for adiponectin in adipose tissue correlated with changes in plasma protein levels of total adiponectin (4). This implies that the alcohol-induced increase in plasma adiponectin levels might be mediated by *de novo* synthesis providing further support that the alcohol itself is responsible for increases in circulating adiponectin levels rather than an alcoholic beverage-specific constituent.

The data revealed that the difference in adiponectin levels between the two treatments are mainly due to a decrease in adiponectin levels during abstention rather than an increase during alcohol consumption. This, and the fairly similar mean HDL-cholesterol levels prior to the interventions compared to after the alcohol intervention, might confirm that the women were indeed habitual drinkers (which was an inclusion criterion for the present study). Furthermore, the ratio of HMW to total adiponectin over the weeks did not differ between treatments. This, also reflected by the high correlations between changes in HMW and total adiponectin, points out that the intervention-induced changes in HMW and total adiponectin levels occur concurrently. Despite different physiological mechanisms, the observed adiponectin-kinetics resemble in part the alcohol-induced increase in apolipoprotein A-I and HDL-cholesterol, although in these cases higher levels were already seen after 5 and 10 days of alcohol consumption, respectively, compared with abstention (26).

In line with previous clinical trials with other groups of young and relatively healthy subjects, we did not observe changes in markers of insulin sensitivity (27;28) or triglycerides levels (29) after moderate alcohol consumption. Studies that did find an effect on insulin levels after alcohol consumption were performed in middle-aged, relatively less insulin-sensitive (3;30) or diabetic subjects (31-33) and lasted between four weeks to up to a year. This suggests that the effect of moderate alcohol intake on markers of glycemia occurs after a longer period of alcohol administration and/or in subjects with (slightly) impaired glucose tolerance rather than in young and insulin sensitive subjects.

Strong points of the study are its randomized crossover design, the high compliance to the treatments throughout the study and the measurement of HMW and total adiponectin with a more sensitive and precise method (Enzyme-linked immunosorbent assay (ELISA)) instead of quantitative western blotting (17) and within the same ELISA (4). Some limitations warrant consideration. The study duration of three weeks of alcohol consumption was relatively short. May be more profound differences would have appeared in adiponectin levels or markers of glycemia if the study persisted longer. Secondly, the women investigated were relatively young and healthy and thus at low risk for type 2 diabetes. However, studies have shown that the association between moderate alcohol consumption and a lower risk of type 2 diabetes also holds in younger women (34) and among subjects already at low risk for diabetes on the basis of multiple combined low-risk lifestyle behaviors (30).

In conclusion, daily moderate alcohol consumption for at least three weeks concomitantly increased total and HMW adiponectin compared with abstention in premenopausal women. Increased circulating adiponectin levels may contribute to a reduced risk for type 2 diabetes in moderate drinkers, not only in postmenopausal women and young and middle-aged men but also among normal-weight, premenopausal women.

Authors' contributions

MMJ provided partial funding, designed and conducted the study, collected, analyzed and interpreted the data and wrote the draft manuscript. RFW helped designing the study and critically reviewed the content for important intellectual content. HFJH provided funding, designed the study and critically reviewed the manuscript for important intellectual content. All authors read and approved the final manuscript.

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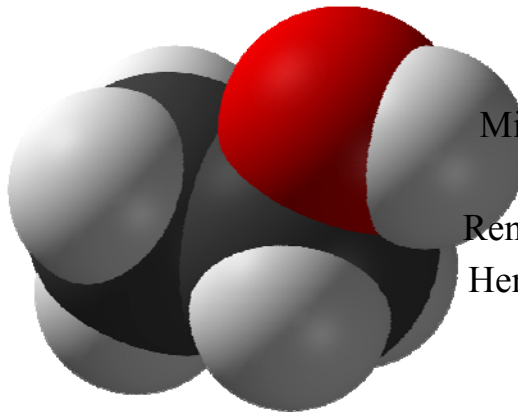
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Chapter 7

Alcohol-induced increase in lipoproteins does not
alter inflammatory response after low-dose *in vivo*
endotoxin challenge in men



Michel M. Joosten
Martine Boetje
Renger F. Witkamp
Henk F.J. Hendriks

Submitted in revised form

Background: Circulating high-density lipoprotein (HDL)-cholesterol and its associated surface lipids apolipoprotein A-I (apoA-I) and phospholipids have been shown to play an important role in the detoxification of lipopolysaccharides (LPS). Moderate alcohol consumption increases HDL-cholesterol, apoA-I and phospholipids.

Objective: to the study was to investigate whether the alcohol-induced increase in lipoproteins attenuates the inflammatory response after a low-dose *in vivo* endotoxin challenge.

Design: In a randomized, crossover trial, 22 men (age: 21-31 y) consumed vodka (30 g/day alcohol) with orange juice or orange juice for four weeks. After each four-week period, subjects were intravenously challenged with a low-dose endotoxin (0.06 ng/kg body weight) bolus. Blood was sampled before and for six hours after the LPS bolus.

Results: Four weeks of vodka consumption increased HDL-cholesterol (10.3%; $P < 0.001$), apoA-I (11.9%; $P < 0.001$), apolipoprotein C-I (7.4%; $P < 0.001$) and phospholipids (7.8%; $P < 0.001$) compared with juice consumption. Inflammatory cytokine and leukocyte responses after the LPS bolus did not differ between treatments. However, tolerance to endotoxin was observed in leukocyte ($P < 0.05$) and TNF- α responses ($P < 0.01$) during the second challenge with LPS.

Conclusions: Despite improved lipoprotein profiles, alcohol consumption did not attenuate the inflammatory response after a low dose *in vivo* endotoxin challenge.

Trial registration: Clinicaltrials.gov ID no.: NCT 00918918

Introduction

In addition to the well-recognized function of lipoproteins in cholesterol and lipid transport, a large body of evidence has demonstrated that lipoproteins also play an important role in the host defense system (1). Lipopolysaccharide (LPS) (also called endotoxin), a component of the gram-negative bacterial cell wall, is among the most potent modulators of the host's innate immune system. Although all lipoproteins have been demonstrated to bind LPS, it mainly binds to high-density lipoprotein (HDL)-cholesterol (60%) in addition to low-density lipoprotein (LDL)-cholesterol (25%) and very low-density lipoprotein (VLDL)-cholesterol (12%) when added to whole human normal blood (2;3). Increasing circulating HDL-cholesterol levels has shown to attenuate the inflammatory effect of endotoxin both in men (4) and mice (5). Conversely, lowering lipoprotein levels rendered animals more susceptible to LPS-induced lethality (6). Studies in both animals and humans suggest that, not HDL-cholesterol itself, but the associated surface apolipoprotein A-I (apoA-I) (7-9) and the phospholipids (2;5;10) are largely responsible for the protective effect against endotoxin-evoked infection. Moreover, increasing phospholipid content of circulating lipoproteins has been shown to decrease the adverse effects of endotoxin in rabbits (11) and mortality in pigs (12).

Moderate alcohol consumption is well-known for its HDL-raising properties, (13;14) which may account for 50% of the protective effect observed in the relation between alcohol consumption and cardiovascular disease (15;16). Besides the increase in HDL-cholesterol, alcohol consumption also increases its associated surface lipids apoA-I, and phospholipids independent of type of beverage (17). Theoretically, this alcohol-induced increase in lipoproteins could increase the scavenging capacity of endotoxin, thereby minimizing the amount of endotoxin available to elicit inflammatory activation.

The injection of a dose of endotoxin into humans has been used for decades as a model to study the interrelationships between infection and the host response (18-20). Using this well-established model of endotoxemia in apparently healthy volunteers, we compared the impact of the alcohol-induced increase in HDL-cholesterol and its associated surface lipids on the sensitivity toward a low-dose *in vivo* endotoxin challenge in men.

Materials and Methods

Study design

The study used a randomized, open label, crossover design, consisting of two four-week periods. Subjects consumed two miniatures (50 ml each) of vodka (Smirnoff, U.K.) (30 g/day alcohol; ~2 drinks according to the U.S. Department of Agriculture (USDA) guidelines (21)) with 200 ml orange juice (Appelsientje, the Netherlands) or only orange juice for four weeks during dinner. The study was conducted at TNO (a Dutch acronym for applied scientific research) Quality of Life, in Zeist, the Netherlands. The study was approved by an independent centralized ethics committee (METOPP; Tilburg, the Netherlands) and is registered at Clinicaltrials.gov, number NCT 00918918. Allocation to

treatment order was randomized according to BMI and age. Blood sampling was done on the morning after four weeks of each treatment period, after an overnight fast.

Compliance was monitored by weekly measurement of urinary ethyl glucuronide (EtG), a direct phase II metabolite of ethanol formed by action of UDP- glucuronosyl transferase. EtG has been reported to be a superior marker with 100% sensitivity as a biomarker of recent drinking. It persists in the urine up to ~75 - 85 h after last intake (22). Cut-off limit to assess compliance during juice intervention for urinary EtG values was set at >0.25 mg/mL (positive sample), to obtain a high sensitivity but avoid positive results due to unintentional ethanol exposure. Cut-off limit during vodka intervention was set at EtG values <0.5 mg/mL (2.2 μ mol/L) (negative sample). Additional measures of compliance were the increase of HDL-cholesterol and the return of empty bottles of vodka.

Subjects

We recruited 24 apparently healthy, non smoking male subjects between 21 and 40 years. Subjects were eligible if they consumed between 5 and 28 units of alcohol per week, were free of any medication at the beginning of the study, and had a BMI between 18 and 27 kg/m² and had no family history of alcoholism. Subjects gave written informed consent and received a compensation for their participation. They were instructed to maintain their habitual body weight, food pattern and physical activity pattern and were told to refrain from any alcoholic products during the entire study except the vodka supplied by TNO.

Experimental protocol

On the morning after each intervention, after an overnight fast, study participants came to the premises. Blood pressure and heart rate were measured by means of OMRON HEM-7080IT-E (Kyoto, Japan). A catheter was inserted in an antecubital vein. At $t = 0$, blood was drawn for baseline measurements. Subsequently, subjects received a bolus infusion of 0.06 ng/kg body weight of endotoxin (*Escherichia coli* lipopolysaccharide, Catalog Number: 1235503, lot G3E069; United States Pharmacopeial Convention Inc, Rockville, MD, U.S.A.) in the antecubital vein. Heart rate and blood pressure were recorded and blood samples were collected at $t = 0, 1, 2, 3, 4, 5$ and 6 hours after endotoxin challenge.

Blood and urine analysis

Blood samples were obtained from the antecubital vein of the forearm and collected in tubes containing clot activator for serum and in ice-chilled tubes containing or Potassium Ethylene Diamine Tetra Acid (K₃EDTA) or lithium heparin for plasma and whole blood (Vacutainer Systems, Becton Dickinson, Plymouth, U.K.). Blood was centrifuged for 15 minutes at 2.000 g at 4°C, within 15 - 30 minutes after collection and stored at <-70°C.

Clinical chemistry, high sensitive C-reactive protein (hsCRP), phospholipids and total leukocytes were analyzed by ADVIA 120/2120 Hematology system (Bayer Diagnostics, U.S.A.). Apolipoproteins in serum were analyzed by bead-based multiplexed immunoassay at the biomarker testing laboratory Rules Based Medicine (RBM; Austin,

Texas, U.S.A.). Tumor Necrosis Factor alpha (TNF- α) was measured by Ultra-Sensitive ELISA (Invitrogen, Paisley, U.K.) and Interleukin (IL)-6 by Luminex using Bio-plex Pro-Assays (Bio-Rad Laboratories, Hercules, CA, U.S.A.), both in plasma according to the manufacturers' specifications. Plasma total adiponectin and lipoprotein binding protein (LBP) were measured using MesoScale Diagnostics assays (Cat# K151IYC-1 and K151BXC-1) (Meso Scale Discovery; Gaithersburg, MD, U.S.A.) and plasma soluble CD14 (sCD14) was measured by means of an immuno-enzymometric (Cat# DC140) (R&D systems; Abingdon, U.K.). EtG was determined was analyzed using a triple quadrupole Ultra Performance LC/MS in MRM mode (Waters, Saint-Quentin en Yvelines, France) as previously described (23).

Statistical analyses

Statistical analyses were performed using the SAS statistical software package (SAS versions 8.2 and 9.1, SAS Institute, Cary, NC, U.S.A.). Variables at the end of the intervention were compared between treatments with a mixed analysis of variance model that included terms for treatment, period and the interaction between period and treatment (indicating possible carryover effects). The inflammatory responses over time between treatments were compared with a mixed analysis of variance model that included terms for treatment, time, period the interaction between time and treatment and the interaction between period and treatment. Correlation coefficients were computed according to Pearson's correlation coefficient or Spearman rank order (for non-normally distributed variables) to assess associations in intervention-induced relative changes between lipoproteins and apolipoproteins and to assess associations between lipoproteins and endotoxin-induced peak response of inflammatory markers. Data are presented as means and SEM unless otherwise specified. Statistical significance was defined as $P < 0.05$.

Results

Intervention

Two of the 24 subjects underwent just one LPS challenge and were excluded for further analyses. Mean age and BMI of the 22 men were 25.0 y (range: 21 - 31 y) and 23.3 kg/m² (range: 19.1 - 27.2 kg/m²). Compliance to treatments was assessed by weekly urinary EtG levels revealing eight positive samples during the juice-drinking period (although all <0.5 mg/mL) and five negative samples during the vodka-drinking period out of the 176 samples analyzed (overall compliance of 93%).

No carryover effects were observed for any outcome measures. HDL-cholesterol significantly increased by 10.3% ($P < 0.001$) after the vodka juice compared to juice consumption. Also, apoA-I (11.6%; $P < 0.001$), apolipoprotein C-I (apoC-I) (7.4%; $P < 0.001$), phospholipids (7.8%; $P < 0.001$) and total adiponectin (11.3%; $P < 0.001$) increased significantly after the vodka juice intervention. Baseline levels of other markers after each four-week period did not differ between treatments (Table 7.1).

Table 7.1: Lipoprotein profile, inflammatory markers, and clinical chemistry at the end of a 4-week intervention period of daily orange juice or vodka (30 g alcohol) with orange juice in 22 young, normal-weight men after an overnight fast.

	Orange juice	Vodka and orange juice	<i>P</i> value [†]
Lipoprotein profile			
HDL-cholesterol (mmol/L)	1.08 ± 0.04	1.18 ± 0.04	< 0.001
Phospholipids (mmol/L)	2.29 ± 0.07	2.46 ± 0.07	< 0.001
ApoA-I (mg/mL)	0.21 ± 0.01	0.24 ± 0.01	< 0.001
ApoC-I (ng/mL)	233 ± 7.8	249 ± 7.8	< 0.001
LDL-cholesterol (mmol/L)	2.57 ± 0.16	2.61 ± 0.12	0.60
ApoB (mg/mL)	1.08 ± 0.06	1.10 ± 0.06	0.56
ApoE (µg/mL)	35.2 ± 2.4	36.7 ± 2.4	0.32
Inflammatory markers			
Total adiponectin (µg/mL)	10.6 ± 1.0	11.8 ± 1.0	< 0.001
TNF-α (pg/mL)	0.93 ± 0.11	0.77 ± 0.11	0.11
IL-6 (pg/mL)	1.24 ± 0.12	1.26 ± 0.12	0.85
hsCRP (mg/L)	0.40 [0.33-1.36]	0.49 [0.24-1.46]	0.50
Total leukocyte count (10 ⁶ /mL)	5.8 ± 0.3	6.0 ± 0.3	0.33
LPS binding protein (µg/mL)	5.3 ± 0.4	5.0 ± 0.4	0.33
Soluble CD14 (µg/mL)	1.23 ± 0.04	1.29 ± 0.04	0.20
Clinical chemistry			
Glucose (mmol/L)	5.30 ± 0.09	5.27 ± 0.09	0.57
Insulin (pmol/L)	60.0 ± 6.9	53.4 ± 6.9	0.33
Triglycerides (mmol/L)	1.19 ± 0.15	1.28 ± 0.15	0.28
Free fatty acids (mmol/L)	0.41 ± 0.03	0.35 ± 0.03	0.13

Values shown are mean ± SEM or median [inter quartile range].

Apo, apolipoprotein;

[†] Mixed ANOVA model on baseline values that included terms for treatment, period and the interaction between period and treatment (to assess carryover effects).

Endotoxin challenge

Endotoxin infusion did not cause any notable adverse events among the subjects. Heart rate and diastolic blood pressure decreased after endotoxin infusion. Heart rate dropped on average from 67 ± 2 beats/min at baseline to 58 ± 2 beats/min at 2 hours whereas diastolic blood pressure dropped from 73 ± 2 mmHg at baseline to 70 ± 2 mmHg at 4 hours (both *P*

< 0.05 for time main-effect). Systolic blood pressure did not change over time but was on average slightly increased after the vodka intervention compared with the juice intervention (117 ± 1 vs. 115 ± 1 mmHg; $P < 0.05$).

When challenged with endotoxin, total leukocyte count and percentage of neutrophils increased significantly ($P < 0.001$) and reached its peak at four 4 after bolus infusion (Figure 7.1). In addition, percentage lymphocytes significantly decreased ($P < 0.001$) after endotoxin infusion to a nadir at 4 hours. The response in circulating total leukocytes or percentages of lymphocytes or neutrophils did not differ between treatments (All $P > 0.45$). However, we observed attenuated total cytokine count ($P < 0.05$) and percentages of lymphocytes and neutrophils (both $P < 0.001$) responses on the second visit after the LPS challenge (Figure 7.1).

The effects on endotoxin infusion on TNF- α and IL-6 levels are shown in Figure 7.2. Both cytokines significantly increased over time ($P < 0.001$) and reached their peak approximately three hours after administration of the LPS bolus. TNF- α ($P = 0.52$) and IL-6 ($P = 0.24$) levels did not differ between treatments. In line with the leukocytes, TNF- α response ($P < 0.01$) was significantly lower during the second time of LPS administration (Figure 7.2). IL-6 response also tended to be lower after the second time, although not significantly ($P = 0.16$).

Correlations

Independent of treatment, serum HDL-cholesterol was tightly correlated with serum apoA-I levels whereas serum LDL-cholesterol was highly associated with serum apoB after each treatment (All $r > 0.82$; $P < 0.0001$). Also intervention-induced percent changes in apoA-I and HDL ($\rho = 0.43$; $P < 0.05$) and apoB and LDL-cholesterol ($\rho = 0.75$; $P < 0.001$) levels were significantly correlated. The relative changes in HDL-cholesterol were also significantly correlated with changes in phospholipids ($\rho = 0.58$; $P < 0.01$) and borderline significant with changes in apoC-I ($\rho = 0.40$; $P = 0.07$) but inversely correlated with changes in apolipoprotein E (apoE) ($\rho = -0.43$; $P < 0.05$).

Due to the significant period effect, correlations between HDL-cholesterol and endotoxin-induced peak levels in inflammatory markers were investigated after the first period only. No correlations were observed between HDL-cholesterol (or associated apolipoproteins) and peak levels of TNF- α at three hours or with IL-6 or total leukocyte count at 4 hours after the LPS bolus at the first challenge.

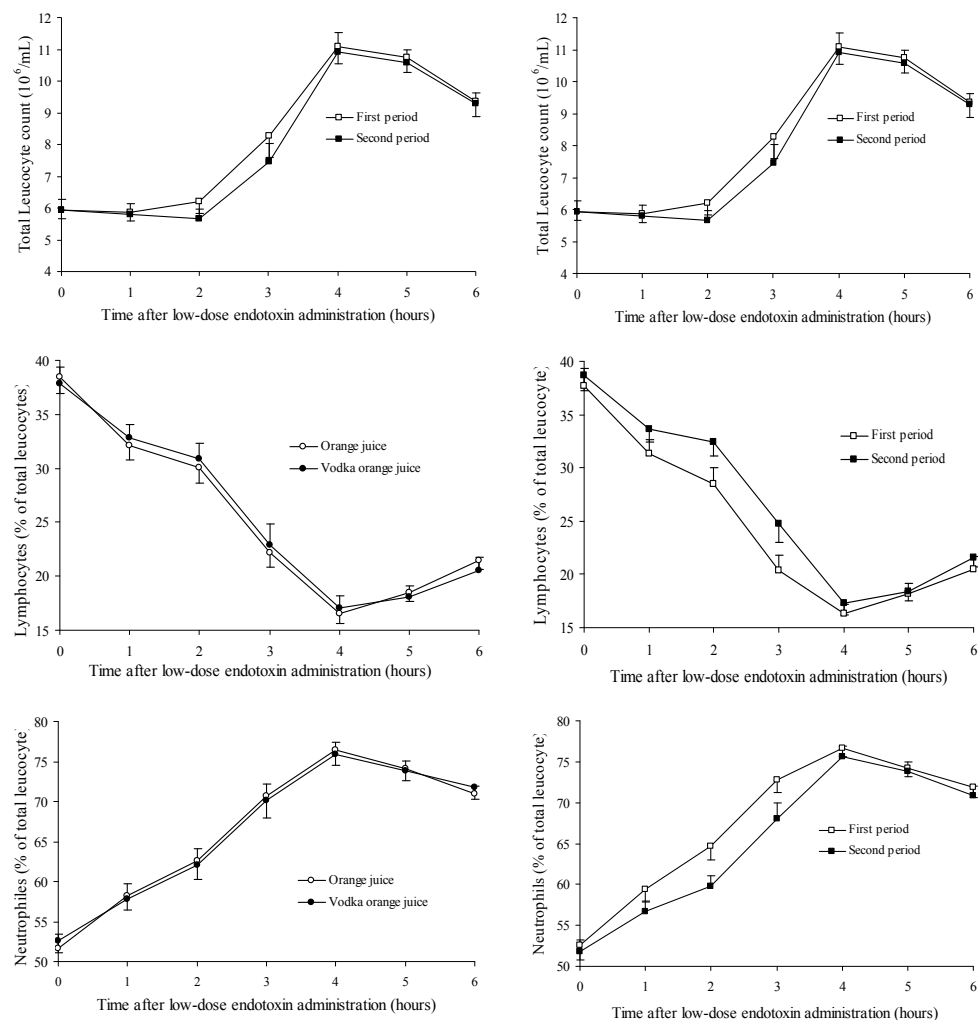


Figure 7.1: Effect of low-dose *in vivo* endotoxin challenge on cytokine response after a 4-week intervention of vodka and orange juice (●) or orange juice only (○) and after the first 4-week intervention (□) or the second 4-week intervention (■) in 22 men. Data are expressed as mean \pm SEM. Responses of total leukocytes count ($P < 0.05$) and percentages of lymphocytes and neutrophils (both $P < 0.001$) were significantly lower during the second period after LPS administration (right panels).

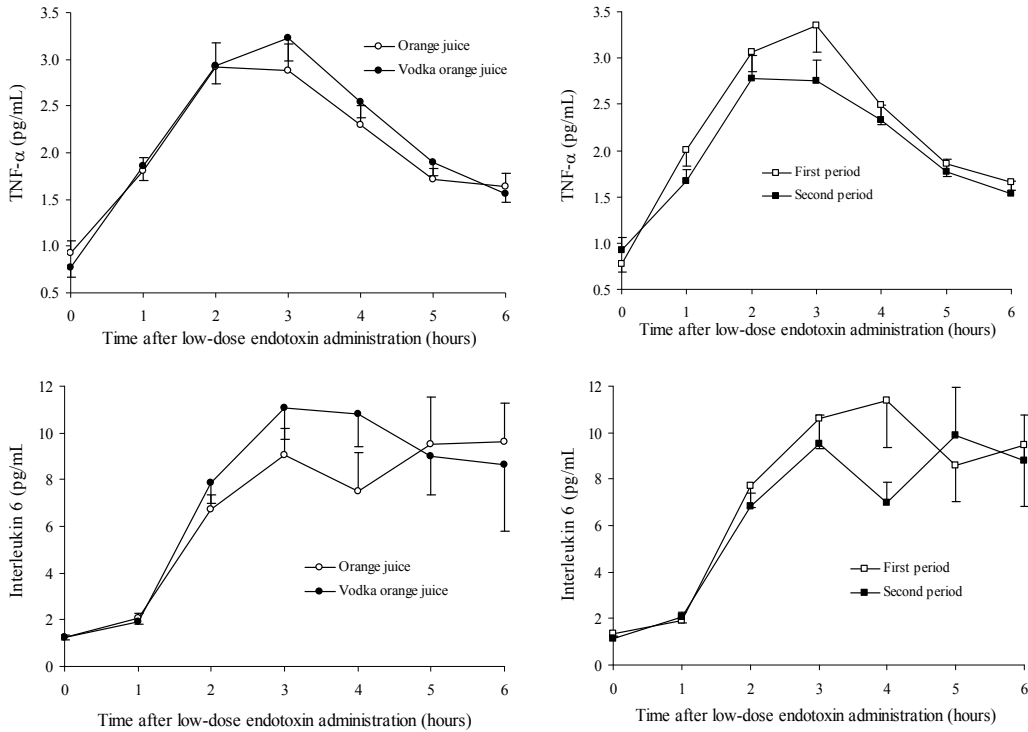


Figure 7.2: Effect of low-dose *in vivo* endotoxin challenge on leukocyte response after a 4-week intervention of vodka and orange juice (●) or orange juice only (○) and after the first 4-week intervention (□) or the second 4-week intervention (■) in 22 men. Data are expressed as mean \pm SEM. TNF- α response was significantly lower ($P < 0.01$) during the second period after LPS administration (upper right panel).

Discussion

After 4 weeks of moderate vodka and juice consumption, HDL-cholesterol, apoA-I, apoC-I, phospholipids and total adiponectin levels were increased whereas LDL-cholesterol, apoB, apoE and several inflammatory markers remained unchanged compared with juice among young, normal-weight men. However, we observed no effect of alcohol consumption on the LPS-induced responses in leukocyte subpopulations or cytokines.

The observations of increased HDL-cholesterol and apoA-I but unaffected LDL, apoB and apoE levels by alcohol consumption are consistent with previous findings in 132 healthy men (24). Previous studies by our group showed similar increases of $\sim 11\%$ in HDL-cholesterol and apoA-I in young men (25) and middle-aged men (14;26) and women (27) after alcohol consumption. Moreover, we noted strong correlations between HDL-cholesterol and apoA-I and between LDL-cholesterol and apoB. Even intervention-induced changes in HDL-cholesterol and apoA-I (26) and LDL-cholesterol and apoB were significantly associated. These findings are in line with a recent meta-analysis among more

than 300,000 people which showed that HDL-cholesterol and apoA-I had very similar shape and magnitude of associations with risk of vascular diseases, as did LDL-cholesterol and apoB (28).

Besides increased HDL-cholesterol and apoA-I, we also found increased levels of apoC-I and total adiponectin. Similar to apoA-I and phospholipids, several human studies suggest that apoC-I can function as part of the innate host defense mechanism against infection. In old age high plasma levels of apoC-I protect against mortality from infection independent of HDL-cholesterol (29). Moreover, apoC-I levels are also correlated with increased survival in patients with severe sepsis (30). In concord with previous findings (23;31;32), we also found higher adiponectin levels after regular moderate alcohol consumption compared with abstinence. Adiponectin levels are inversely associated with markers of inflammation (33;34). Moreover, it has been shown that adiponectin exerts anti-inflammatory effects via macrophages, suppressing the production of pro-inflammatory cytokines in response to LPS (35).

Despite the increases in HDL-cholesterol and several associated surface markers important for LPS detoxification, the inflammatory response after the LPS bolus did not differ between treatments. Possibly, a potential anti-inflammatory effect could have counteracted by a pro-inflammatory effect after moderate alcohol consumption. Concentrations of increased lipopolysaccharide-binding protein (LBP), soluble CD14 (sCD14) and endotoxin levels (36), suggestive of increased permeability or 'leaky gut' (37), and a decreased endotoxin-binding capacity of whole blood (38) have been reported in persons with chronic alcohol misuse. Both LBP and CD14 play a role in the response to gram negative compounds depending on their concentration and environment (39;40). For example, they transfer LPS to the signaling receptor complex MD-2/Toll-like receptor 4 (TLR4) on macrophages. However, plasma LBP or sCD14 levels were unaffected by moderate alcohol consumption. Also, in observational research moderate amounts of alcohol, comparable as used in our study, were not associated with endotoxemia in apparently healthy men (41).

Alternatively, the unaltered response after LPS administration between treatments might be due to tolerance to the endotoxin. Peak and averaged values of total leukocyte and cytokine responses were lower the second administration compared to the first administration. These are strong indications for tolerance. Although tolerance to LPS has been described before in humans after days (42;43) or 2 weeks (44), it has never been described as late as 4 weeks after a previous challenge. This tolerance could have obscured potential treatment effects due to the randomized crossover design of our study. We compared treatments taking into account results after the first LPS administration period only. This did not materially change our findings (data not shown). However, our study was not designed for such a comparison and was therefore highly underpowered.

Regardless of possible increased anti-inflammatory properties of HDL-cholesterol and its associated surface lipids, a functional consequence of the alcohol-induced elevation in HDL is an increased cholesterol efflux. The improved cholesterol efflux is mediated by

stimulating early steps in reverse cholesterol transport (17) and by ATP-binding cassette transporter protein A1 (ABCA1) (26). Furthermore, increased paraoxonase (PON) activity has been observed after moderate alcohol consumption (14;27). PON, an enzyme entirely complexed to HDL-cholesterol, may protect against atherosclerosis by its anti-oxidative properties (45).

Experimental endotoxemia can provide unique insights into the relationship of inflammation and metabolic disturbances (20;46). Nevertheless, several limitations of this model warrant consideration. Based on previous research that showed HDL-mediated decreases in inflammatory responses in humans (4;7;10), we chose LPS as an inducer of inflammation. It should be noted that endotoxin is just one of many ways to activate the immune system. Second, the dose of endotoxin might have been too low to pick up differences in HDL-cholesterol's anti-inflammatory potential, compared with previous research where they used 1-4 ng/kg body weight (4;7;19;46). Fourth, structural differences in LPS derived from different strains exist. However, both the dose (0.06 ng/kg bodyweight) and strain of LPS have been used before (44;47) and give physiological relevant rather than supra physiological elevations of TNF- α . With this model we succeeded to mimic a more naturally occurring endotoxemia, as seen in humans e.g. after a high fat meal (48). Finally, we induced a more acute rather than chronic low-grade inflammation. Especially the latter has been proposed to be associated with several diseases states. Therefore, low-dose endotoxin administration for a longer period might more closely mimic this low-grade inflammatory state.

To summarize, moderate alcohol consumption (approximately two to three drinks a day) for four weeks increases serum HDL-cholesterol, apoA-I, apoC-I and phospholipids, which all have been linked to play important roles in the detoxification of LPS. This improvement of lipoprotein profile did not attenuate the inflammatory response after a low dose of LPS bolus, possibly due to tolerance to the endotoxin. Whether the increase in HDL-cholesterol and its associated surface lipids after moderate alcohol consumption has potent anti-inflammatory properties *in vivo* warrants further clarification.

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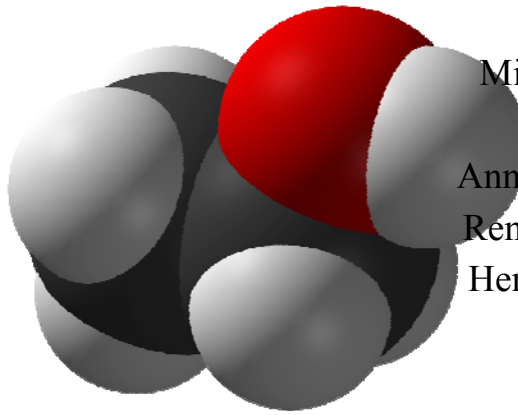
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Chapter 8

Short-term oral exposure to white wine transiently
lowers serum free fatty acids



Michel M. Joosten
Cees de Graaf
Annemarie Rietman
Renger F. Witkamp
Henk F.J. Hendriks

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Background: Preingestive or cephalic phase responses, triggered by sensory stimulation of nutrients, influence the organism's digestive and endocrine responses and substrate mobilization. Whether oral sensory stimulation with alcohol elicits cephalic phase responses is uncertain.

Objective: To determine whether oral alcohol exposure, in the form of white wine, provokes cephalic phase responses in normal-weight and overweight subjects.

Design: In a semi-randomized, crossover trial, eleven normal-weight (BMI 19-25 kg/m²) and eleven overweight (BMI 27-35 kg/m²) postmenopausal women sham-fed, after an overnight fast under three separate conditions four weeks apart, 41 g cake (750 kJ), 25 cL white wine (750 kJ; ~26 g alcohol) and 25 cL water. Blood was drawn prior to and for 30 min after two 3-minute episodes of modified sham feeding (MSF). Blood samples were analyzed for free fatty acid (FFA), triglyceride, glucose, pancreatic polypeptide (PP), insulin and alcohol concentrations.

Results: Incremental area under the curves (IAUC) of FFA concentrations differed significantly ($P < 0.001$) between the three treatments but not between BMI categories ($P = 0.11$). After MSF with white wine, FFA concentrations dropped to a minimum of $77 \pm 3\%$ of baseline concentrations at $t = 12 \pm 2$ min after baseline and returned to baseline after ~30 min, whereas after MSF with cake and water FFA concentrations gradually increased. IAUC of triglycerides, glucose, PP and insulin concentrations did not differ between the three treatments (All $P > 0.05$).

Conclusions: Short-term oral white wine exposure substantially and temporarily decreases FFA concentrations suggesting a cephalic phase response of alcohol. This effect occurred regardless of BMI.

Trial registration: Clinicaltrials.gov ID no.: NCT00652405.

Introduction

The digestion of food is generally being divided into the cephalic, gastric, and intestinal phases. Cephalic phase responses are physiologic changes in response to food cues such as thought, smell, sight and taste of palatable food. These preingestive responses are rapid (occurring within minutes after stimulation), transient (returning to near baseline within minutes) and relatively small compared to the magnitude achieved when food is actually being ingested (1). The main function of the cephalic phase may be essentially adaptive, preparing the digestive system for the reception, digestion and absorption of the nutrients from food (2).

The primary metabolic changes occurring during this phase are gallbladder contraction (3), gastric acid production (4), and stimulation of both the exocrine and endocrine pancreas (5). Its physiological relevance in response to meal ingestion becomes apparent when blocking (6) or bypassing (7) the cephalic phase, which has detrimental effects on postprandial glycemia and insulinemia. Moreover, modified sham feeding (MSF), a method to provoke cephalic phase responses, with fatty foods alters mobilization of free fatty acids (FFA), triglycerides and glucose in the postprandial state (8-12). These effects are absent when sham feeding with water (9;11).

Cephalic phase responses are thought to be mediated by the efferent component of the vagus nerve, which in turn induces changes in substrate utilization. Vagal stimulation can be measured indirectly by pancreatic polypeptide (PP) release after MSF (13). Sham feeding with foods rich in protein and fat and carbohydrates (13;14) indeed exert cephalic phase PP responses. Furthermore, oral fat (9;11;12) and carbohydrate exposure (15) have been shown to induce a cephalic phase insulin release.

Whether and to what extent tasting alcohol affects cephalic phase responses remains unclear. Since the cephalic phase plays a role in provoking physiological changes pivotal for food digestion, appetitive sensations (11;12) and food intake (14) it is important to know whether alcohol could exert a cephalic phase response (CPR). With this study we therefore wanted to investigate the effects on substrate mobilization and hormone release after oral white wine exposure.

Methods

The study used a semi-randomized, open-label, crossover design. It was part of an intervention in which subjects daily consumed white wine (~70 kcal/100 mL, ~26 g alcohol; Droë Steen; South Africa), followed by mineral water (Vittel, France) or vice versa. Subjects consumed these study substances for four weeks during dinner. Allocation to treatment order was randomized according to BMI category. The study was conducted at TNO (a Dutch acronym for applied scientific research) Quality of Life, in Zeist, the Netherlands. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by an independent centralized ethics committee (METOPP; Tilburg, the Netherlands). Written

informed consent was obtained from all subjects. This trial is registered at clinicaltrials.gov as NCT00652405.

Subjects

We recruited 24 nonsmoking women from a pool of volunteers of TNO Quality of Life. We chose postmenopausal women to prevent possible effects of the menstrual cycle on CPR. Eligible subjects were apparently healthy, free of any medication use, had an absence of menses for at least 2 years, a BMI between 19 and 25 for normal-weight or 27 and 35 kg/m² for overweight subjects and no family history of alcoholism. The overweight group had a BMI of 27 and higher to increase the potential contrast between the two BMI groups. BMI groups were matched for age and restraint eating score on the Dutch Eating Behavior Questionnaire (16) as differences may exist in cephalic phase response between restraint and unrestraint eaters (17).

Experimental protocol

Each subject was subjected to three experimental conditions 4 weeks apart from each other. The conditions were administered in a semi-counterbalanced order after a 10 h overnight fast: (a) sham-feeding cake (positive control), (b) sham-feeding 25 cL of water (negative control) and (c) sham-feeding 25 cL of white wine (13% vol). For logistic reasons the cake condition was always first. The order of sham feeding water or wine was the same as the treatment order of the intervention. All three stimuli were served at room temperature (isothermic). The energy content of the 41 g cake (Albert Heijn, The Netherlands; 5% protein, 41% carbohydrates and 54% fat) equaled the energy content of the 25 cL of white wine (~100% alcohol). High fat cake was chosen as it has been demonstrated to elicit a cephalic phase PP release (14).

Each test morning, an indwelling cannula was placed in the forearm for venous blood draws. During the experimental days subjects took, at $t = 0$ min (baseline), a bite or sip of the food, oral processed it without swallowing for as long as the stimulus felt comfortable in their mouth, and then expectorated the stimulus. Subjects repeated this procedure for a 3-min period in which half of the stimulus portion size was sham-fed. In a second 3-min period, from $t = 4$ min onwards, the other half of the stimulus was sham-fed. Taken together, a 6 min-stimulation period with a one min break for blood sampling was given. After each 3-min sham feed period, subjects were allowed to rinse their mouth once with water. Stimuli were weighed (Mettler Toledo, Switzerland) before and after sham feeding to check whether subjects had accidentally swallowed parts of the sham feed stimuli. Blood sampling for FFA, triglycerides glucose, PP and insulin took place at $t = 0, 4, 8, 12, 16, 20$ and 30 min. At $t = 8$ min additional blood and saliva were sampled to determine alcohol concentrations. Unstimulated saliva (to avoid possible CPR due to chewing) was collected for four minutes after $t = 8$ min.

Blood and saliva sample analysis

Blood samples were obtained from the antecubital vein of the forearm and collected in tubes containing clot activator for serum and in ice-chilled tubes containing or Potassium Ethylene Diamine Tetra Acid (K₃EDTA) for plasma (Vacutainer Systems, Becton Dickinson, Plymouth, U.K.). Blood was centrifuged for 15 minutes at 2.000 g at 4°C, within 15 - 30 minutes after collection and stored at -80 °C. FFA, triglyceride, glucose and insulin determinations in serum were performed using Olympus analytical equipment and reagents. PP determinations in serum were performed using radioimmunoassay by using a commercially available kit (Euro-diagnostica, Sweden) with a detection limit of 3.1 pmol/L and intra-assay and inter-assay coefficient variations of 3.5% and 2.6% respectively. Alcohol concentrations in plasma and saliva were determined by headspace gas chromatography (18). The detection limit of the alcohol concentration in blood and saliva was 0.1 mmol/L. Concentrations below the detection limit were set at half the detection limit.

Statistical analyses

The rationale for the sample size was based on a previous study in women with a very similar experimental design to elicit a cephalic phase PP release (14). Cumulative changes in serum FFA, triglycerides, glucose, PP, and insulin responses were quantified as the incremental (delta from baseline) area under the 30 min time-concentration curve using the trapezoidal rule. Baseline values and incremental area under the curves (IAUCs) of each metabolite/hormone and alcohol concentrations were compared with a mixed analysis of variance (ANOVA) model that included terms for treatment, BMI category and their interaction. If a significant treatment effect occurred, a post hoc Tukey test was used and for IAUCs summary statistics such as time to peak / nadir (and percentage difference from each subject's own baseline value (to compensate for baseline differences between normal-weight and overweight subjects) were computed. In case of a significant treatment effect, a responder was defined as having an increase or decrease of >14% from baseline serum concentration at two consecutive time points within 16 min after initiation of MSF. We used SAS statistical software package (SAS version 8, SAS Institute, Cary, NC, U.S.A.) to perform the statistical analyses. *P* values < 0.05 were considered statistical significant. Results are presented as mean (± SEM) unless otherwise specified.

Results

One normal-weight subject dropped out due to study-unrelated reasons, one overweight subject was left out of the analysis as she had swallowed parts of the sham feed stimuli on all three occasions. Overweight subjects had a significant higher BMI (mean ± standard deviation: 29.8 ± 2.6 vs. 22.8 ± 1.4 kg/m²; *P* < 0.0001) but did not differ in age (55.6 ± 3.0 vs. 56.0 ± 2.7 y; *P* = 0.82) or restraint eating score (2.7 ± 0.7 vs. 2.7 ± 0.7; *P* = 0.90) compared with normal-weight subjects respectively.

Table 8.1: Serum concentrations of metabolites and hormones at baseline (t = 0) before modified sham feeding cake, water or white wine in normal-weight (N = 11) and overweight (N = 11) postmenopausal women after an overnight fast.

	Cake		Water		White wine	
	Normal-weight	Overweight	Normal-weight	Overweight	Normal-weight	Overweight
Metabolites						
Free fatty acids (mmol/L) [†]	0.47 ± 0.06	0.72± 0.06	0.44 ± 0.06	0.62± 0.06	0.49 ± 0.06	0.65± 0.06
Triglycerides (mmol/L)	1.22 ± 0.22	1.71 ± 0.22	1.36 ± 0.22	1.70 ± 0.22	1.43 ± 0.22	1.70 ± 0.22
Glucose (mmol/L) [†]	5.27 ± 0.13	5.65 ± 0.13	5.19 ± 0.13	5.57 ± 0.13	5.29 ± 0.13	5.73 ± 0.13
Hormones						
Pancreatic polypeptide (pmol/L)	28.7 ± 8.0	24.1 ± 8.0	26.8 ± 8.0	19.8 ± 8.0	23.7 ± 8.0	28.4 ± 8.0
Insulin (pmol/L) [†]	39.8 ± 6.9	64.0 ± 6.9	44.3 ± 6.9	59.6 ± 7.1	39.1 ± 6.9	65.2 ± 6.9

Data are expressed as mean ± SEM. No interaction effects for treatment x BMI category or main effects for treatment at baseline were observed in metabolites and hormones, all $P > 0.05$ (Mixed ANOVA on baseline values).

[†] Significant main effect for BMI category, $P < 0.05$ (Mixed ANOVA on baseline values).

At baseline ($t = 0$ min) there were no differences in values of metabolites and hormones between the three treatments (Table 8.1). Overweight subjects had higher levels of FFA, glucose and insulin compared with normal-weight subjects at baseline (All $P < 0.05$).

Expectorated stimuli weights and alcohol concentrations

Stimuli weights after MSF increased by a mean of 13.9 ± 1.3 g after sham feeding cake, 15.8 ± 1.9 g after sham feeding white wine and 0.2 ± 1.3 g after sham feeding water due to the added weight of the saliva.

At $t = 8$ min, mean saliva (SAC) and blood alcohol concentrations (BAC) differed significantly between treatments (both $P < 0.0001$) but not between BMI categories (both $P > 0.80$). SAC and BAC after wine exposure reached a mean of 152 ± 13 mmol/L and of 0.16 ± 0.02 mmol/L (Range: $<0.1 - 0.44$ mmol/L) respectively whereas no detectable alcohol levels were found after cake and water exposure (all <0.1 mmol/L). Three women (one normal-weight and two overweight women) had plasma alcohol levels below the detection limit (<0.1 mmol/L) at $t = 8$ min after MSF with white wine.

Metabolites

Mean serum FFA concentrations dropped after MSF with white wine and returned to near baseline values after MSF stopped whereas FFA concentrations after sham feeding cake and water resulted in a gradual increase over the 30 min time span (Figure 8.1). IAUC of serum FFA concentrations differed significantly ($P < 0.001$) between the three treatments. Post hoc analyses showed that IAUC of FFA after MSF with white wine differed significantly from IAUC of water ($P < 0.001$) and cake ($P < 0.001$), whereas water and cake did not differ ($P = 0.79$). The nadir of FFA concentrations after MSF white wine reached its minimum at $t = 12 \pm 2$ min after baseline (12 ± 2 min for normal-weight and 12 ± 2 min for overweight women). At this time point, mean FFA concentrations dropped to $77 \pm 3\%$ of baseline values ($79 \pm 5\%$ for normal-weight and $74 \pm 4\%$ for overweight women). Eighty-two percent (18/22; 9 normal- and nine overweight subjects) of the women could be marked as a responder after oral alcohol exposure (based on a drop of $>14\%$ in basal FFA concentration at two consecutive time points within 16-min after MSF). The effect even occurred in two of the three subjects who had no detectable blood alcohol concentrations at $t = 8$ min. Only 5% (1/22) and 0% (0/22) of the subjects showed such a decrease after orosensory stimulation with water and cake respectively. No main effect for BMI category ($P = 0.11$) or interaction effect for BMI category \times treatment ($P_{\text{interaction}} = 0.35$) were observed (Figure 8.1).

Mean serum triglyceride concentrations after the three MSF treatments remained close to fasting concentrations (Figure 1). IAUC of serum triglycerides concentrations did not differ between the three treatments ($P = 0.66$) and BMI categories ($P = 0.72$). Also no interaction effect for BMI category \times treatment ($P_{\text{interaction}} = 0.51$) was observed.

Mean serum glucose concentrations after the three MSF treatments remained close to fasting concentrations (Figure 8.1). IAUC of serum glucose concentrations did not show

a treatment ($P = 0.69$) or BMI category \times treatment effect ($P_{\text{interaction}} = 0.63$). However, overweight women had lower glucose IAUC after all three MSF treatments compared to normal-weight women ($P = 0.03$).

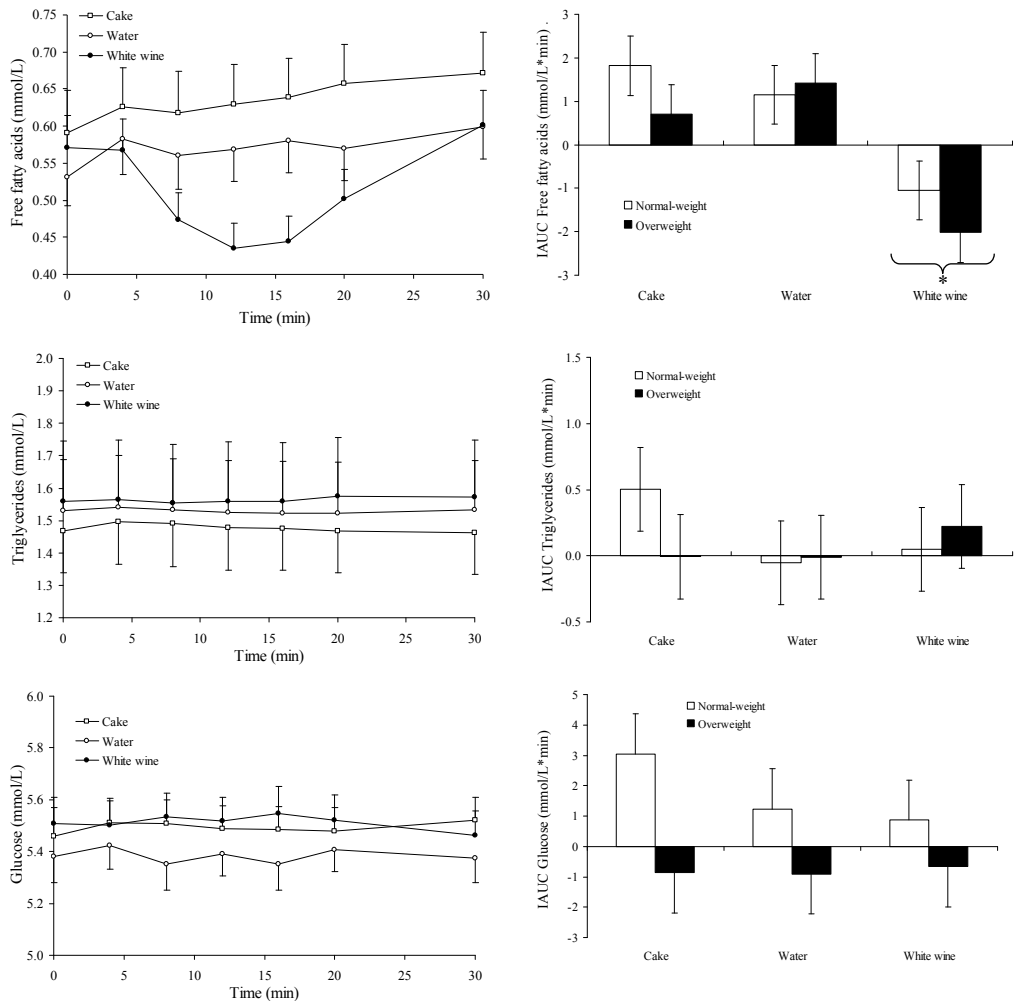


Figure 8.1: Mean (\pm SEM) serum free fatty acid, triglyceride and glucose response curves in women ($N = 22$) and incremental area under the curve (IAUCs) in normal-weight ($N = 11$) and overweight ($N = 11$) women after modified sham feeding (MSF) cake, water or white wine for two 3-minute episodes.

* Significantly different from water and cake, $P < 0.001$ (Mixed ANOVA on IAUCs, followed by a post hoc Tukey test).

Significant main effect for BMI category in glucose IAUCs, $P = 0.03$ (Mixed ANOVA on IAUCs).

Hormones

Three women (one normal-weight and two overweight women) had PP concentrations below the detection limit of the assay throughout all three test conditions. Excluding them from the analysis did not essentially change the results. Therefore, unadjusted results are presented here.

Mean serum PP response curves after the three treatments showed increased PP concentrations towards the end (at $t = 30$ min) after all three MSF procedures (Figure 8.2). IAUC for serum PP concentrations did not differ between the three MSF treatments ($P = 0.38$). Also no BMI category effect ($P = 0.51$) or BMI category \times treatment interaction ($P_{\text{interaction}} = 0.71$) were observed.

Mean serum insulin response curves after the three MSF treatments remained close to fasting concentrations (Figure 2). IAUC for serum insulin concentrations did not show a treatment ($P = 0.22$), BMI category ($P = 0.84$) or BMI category \times treatment interaction effect ($P_{\text{interaction}} = 0.28$) (Figure 8.2).

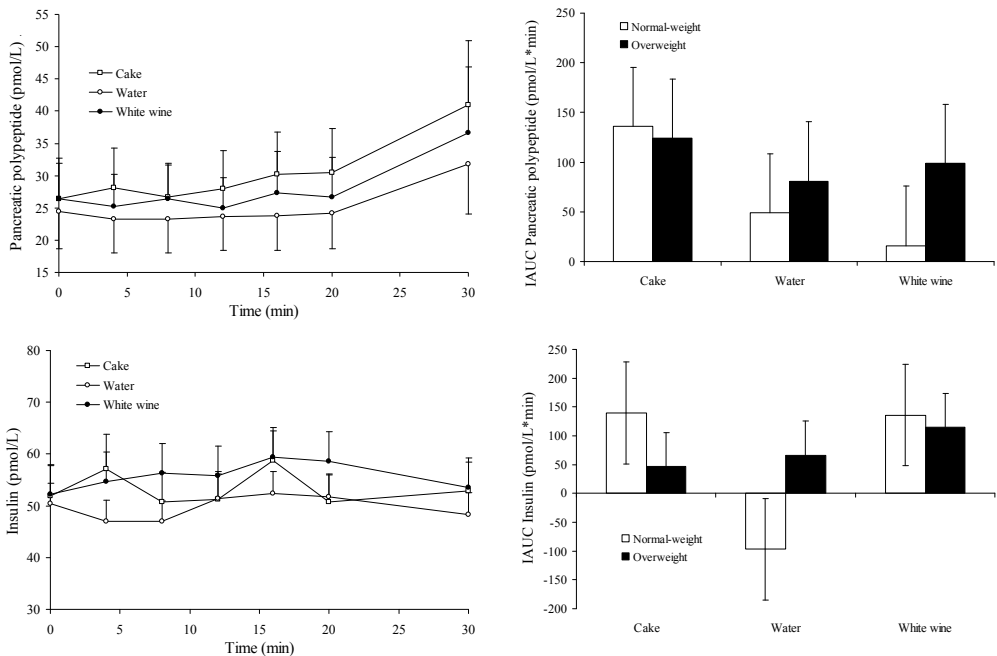


Figure 8.2: Mean (\pm SEM) serum pancreatic polypeptide (PP) and insulin response curves in women ($N = 22$) and incremental area under the curve (IAUCs) in normal-weight ($N = 11$) and overweight ($N = 11$) women after modified sham feeding (MSF) cake, water or white wine for two 3-minute episodes.

No main or interaction effects, $P > 0.05$ (Mixed ANOVA on IAUCs).

Discussion

The present study demonstrates that short-term oral exposure to white wine substantially and robustly decreases serum FFA concentrations, irrespective of BMI. This effect was rapid (occurring within minutes of MSF), transient (returning to baseline after ~20 min when MSF stopped) and small (relative to the magnitude achieved when alcohol is actually being ingested; 23% reduction compared to 47% reduction (19) in FFA concentrations), indicating a cephalic phase response (1). No alterations were observed in other metabolites or hormones.

To further ascertain that the decrease in FFA concentrations was a cephalic effect rather than a gastric or intestinal effect, several additional markers were evaluated. The weight, albeit crude, before and after mastification was determined, which indicated that little or none of the white wine was inadvertently swallowed during MSF. Furthermore, alcohol levels in saliva and plasma were measured. Despite a very small increase in mean plasma alcohol concentrations they remained ~60 times lower compared to ingestion of a similar amount of alcohol (19). Furthermore, triglyceride and glucose concentrations remained close to fasting concentrations after MSF with wine (Figure 8.1). It is well-documented that acute alcohol ingestion not only lowers FFA (19-22) but also strongly elevates triglyceride (19;23-25) and lowers glucose concentrations (23;26). We did not notice an increase in triglyceride or a decrease in glucose concentrations after MSF with alcohol. It thus seems unlikely that the relatively low and clinically irrelevant blood alcohol concentration could have evoked this substantial drop in FFAs without affecting triglycerides or glucose.

Despite evidence of a substantial decrease in FFA concentrations after MSF with wine, we did not document any changes in vagal activity. This is in line with a recent study in which MSF with both sweet and salty solutions did not increase PP (27). The lack of significant increases in cephalic phase PP levels in response to liquid stimuli suggests that in humans, chewing may be a required stimulus for vagal activation. However, also after MSF with cake, a stimulus that needs to be chewed, no significant increase in PP release was observed in our study. Prior observations showed that chewing gum, whether unflavored or flavored with a non-nutritive sweetener or the sweetener paired with a mint flavor, also did not significantly increase PP levels (27). Perhaps the amount of MSF stimuli might have been too little (14) and or the duration of MSF might have been too short (12;28) for a significant PP release. In three women serum PP concentrations even failed to reach the detection limit of the assay throughout all three treatments. This finding is in accordance with observations of others where healthy adults also had PP concentrations below the detection limit of the assay (29). Furthermore, in another study some men and women even lacked PP responses after swallowing a meal (13). We observed increased PP concentrations at the end of all three treatments in our study. This indicated that PP secreting cells were intact in our subjects and it also provided evidence that the analytical method was able to measure changes in PP concentrations.

Whatever the mechanism from wine in the oropharyngeal cavity may be, it must elicit responses that increase FFA absorption from the bloodstream or decrease FFA supply from the adipose tissue to account for the decline in FFA concentrations after oral alcohol exposure. Increased storage of FFA is under control of acylation-stimulating protein (ASP). This stimulates storage of free fatty acids (FFA) as triglycerides in adipose tissue, but also increases glucose uptake (30). Since no changes in glucose were observed after MSF with wine, it seems unlikely that increased ASP activity could have explained this finding. A decreased supply of FFAs via lipoprotein lipase, out of chylomicron triglycerides is not very likely to be the cause, as this action predominantly occurs in the postprandial period (31). A plausible mechanism may be due to the altered activity of hormone-sensitive lipase (HSL), an enzyme responsible for hydrolysis of stored triacylglycerols in adipose tissue (32). HSL activity in adipose tissue is known to also be under control of the vagal nerve (33). Lowered activity of HSL results in a reduction in the rate of FFA release from adipose tissue stores.

Given the facts that the drop in FFA is preingestive and that the FFA response curve shows all characteristics of a cephalic phase response based on the magnitude and time span in which the effect occurred one can consider the effect cephalic rather than a gastric or intestinal. Furthermore, irrespective whether the effect is a 'true' cephalic phase effect or not, it still is of physiological relevance as the decrease in FFAs was substantial, robust and it occurred after stimulation durations likely to be encountered in normal drinking (~6 min). It implies that mere oral wine exposure without ingestion already triggers metabolism and may serve as a feed-forward system to optimize the absorption and utilization of dietary constituents such as alcohol.

As cephalic phase responses are known to affect satiation (34) and satiety (11;12) the sudden drop in FFA after oral wine exposure might possibly be associated with increased appetite sensations and energy intake as observed after alcohol consumption (35).

The acute drop in FFAs after oral exposure might also improve insulin sensitivity as observed after both acute alcohol consumption (21) and prolonged consumption (36). Acute decreases in serum FFA concentrations are directly related to improved insulin signaling (37) and glucose metabolism (38). It has been proposed that acute FFA reductions could reflect the improved insulin sensitivity observed after acute moderate alcohol consumption (21;39).

This study has only been executed with white wine. Whether similar findings will be found with other alcoholic beverages has yet to be investigated. It seems likely, however, that this would be the case for several reasons. FFA concentrations are decreased after acute alcohol consumption per se (19-22), and not only after white wine consumption. Secondly, other physiological effects described after alcohol consumption (e.g. increase in HDL-cholesterol (40)) are observed for all three types of alcoholic beverages.

Strong points of the study are its crossover design, its relatively large and homogenous group of subjects and the incorporation of both hormones and metabolites. Several limitations warrant consideration. The white wine differed in structure (liquid vs.

solid compared to the cake) and energy content (compared to water). However, differences in structure or amount of calories between stimuli are unlikely to have caused this robust and substantial drop in FFA concentrations after MSF with wine since both after sham feeding cake and water FFA concentrations gradually increased; a process that naturally occurs with prolonged fasting. Another control might have been a non-alcoholic wine or ethanol in water. We choose cake rather than an ethanol-related control solely to have an expected positive control for cephalic phase PP release.

We conclude that mere oral exposure to white wine is capable of reducing serum free fatty acid concentrations, regardless of BMI. Furthermore, this study demonstrates that stimulation durations likely to be encountered in normal drinking are sufficient to elicit such a temporarily but substantial and robust response. Further study of the cephalic phase response of alcohol, also in other beverages, on its physiological implications such as appetite and insulin sensitivity is warranted.

Authors' contributions

MMJ designed the protocol, interpreted the data, and drafted the manuscript. MMJ and AR performed the statistical analysis. CdG and RFW provided significant advice during designing the protocol and writing of the manuscript. HFJH was involved in the design of the protocol, provided significant advice during writing of the manuscript and obtained funding. The authors have no conflict of interest with respect to the work described in the manuscript.

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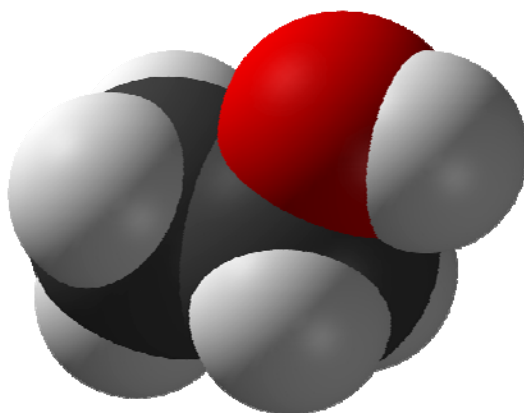
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Chapter 9

General discussion



The research described in this thesis aimed to contribute to a better understanding of the association between alcohol consumption and type 2 diabetes risk that has previously been found. To this end, two main research strategies were followed. First observational studies were applied to extend the evidence of this relation. Second, underlying physiological mechanisms were further investigated in controlled study settings.

This final chapter starts with a brief overview of the main findings, followed by a section on methodological considerations, a reflection of the findings and comparison with other studies. Finally, implications for public health and recommendations for future research are given.

Main findings

The main findings of the observational and experimental studies conducted and described within this thesis are summarized in Table 9.1.

Prospective cohort studies

The effect of change in alcohol consumption over time on diabetes risk depended on initial consumption level in U.S. middle-aged men. Only among initially rare and light drinkers, increases in alcohol consumption over time were associated with increased levels of total adiponectin and with a lower risk of type 2 diabetes. When considering these endpoints, men already consuming alcohol in moderation did not gain further health benefit from increased consumption. In line with data obtained from the general population, we found that moderate alcohol consumption is associated with a lowered risk of type 2 diabetes in Dutch men and women already at lower risk due to favorable lifestyle characteristics. In these subjects who carry a relatively low risk of developing diabetes on the basis of other lifestyle characteristics, alcohol consumption provided a considerably further decrease (about 40% or more) in the risk of diabetes.

Randomized controlled trials

The randomized intervention studies carried out showed that moderate alcohol consumption increased both total and high-molecular weight adiponectin levels by about 10% compared to abstention. These effects were not depending on beverage type (beer, wine or spirits), age (young and middle-aged subjects) or gender. Moreover, the increase in adiponectin concentrations could be attributed to increased *de novo* syntheses in adipose tissue as intervention-induced changes in total adiponectin protein levels correlated to changes in expression of the gene encoding adiponectin. Furthermore, the weekly alterations in both total and HMW adiponectin occurred concomitantly and were evident within three weeks of moderate alcohol consumption compared with abstention.

Among postmenopausal women, we observed favorable changes in fasting insulin and triglyceride levels after six weeks of moderate alcohol consumption but not in younger women and men after three and four weeks, respectively. An integrated approach of large-

scale profiling of proteins and genes revealed that moderate alcohol consumption for four weeks altered gene expression profiles of white blood cells and circulating proteins both related to immune response and lipid metabolism. However, we could not confirm that four weeks of moderate alcohol consumption resulted in a lower inflammatory response induced by a low dose *in vivo* endotoxin bolus, despite a 10% increase in HDL-cholesterol and several HDL-associated apolipoproteins in men. Finally, we found that short-term oral exposure of white wine without swallowing provoked a substantial decrease in circulating free fatty acid concentrations in normal-weight and overweight postmenopausal women. The free fatty acid concentrations returned to near baseline values in less than half an hour after the exposure to the wine stopped.

Methodological considerations

This section discusses methodological considerations which are important to take into account when interpreting the results. The research described in this thesis consisted of prospective cohort studies and randomized controlled trials, both subject to certain strengths and limitations. Although these have been pointed out in the discussion sections of each chapter, several general but iterative aspects will be briefly commented on to put the observed findings into perspective.

Prospective cohort studies

Compared with randomized trials results of prospective cohort studies can be easily generalized as they are based on the natural development of a trait in a real-life setting. The observational studies described in this thesis had several strengths, such as a prospective and long follow-up, large samples sizes, and verified cases of a hard end point: incident type 2 diabetes. However, there are, as with any prospective studies, certain limitations. In the following section some limitations specifically of interest in prospective studies on alcohol consumption will be addressed.

Reference group

One of the concerns related to observational studies with alcohol is also known as the "sick-quitter" hypothesis. It was proposed by Shaper and colleagues in 1988 (1) and substantiated later (2;3). This hypothesis argues that abstainers are an inappropriate control population because at least some of these people may abstain because of illness or former alcohol abuse or because alcohol interacts with prescription drugs they are taking. Obviously, comparisons of healthy drinkers with abstainers who take prescription drugs or who have underlying illnesses that raise one's risk for chronic diseases will produce a biased result in favor of the alcohol-consuming subjects. Because an individual's health status informs such a decision, comparing current with former drinkers can be biased. This "sick-quitter" effect may also explain a small portion of the benefit attributed to alcohol in some studies on type

Table 9.1: Summary of the main findings of the prospective cohort studies and randomized crossover trials described in this thesis.

Chapter	Population	Duration	Beverage type and amount	Outcome	Measure	Estimate (95% CI) ¹
Prospective cohort studies						
2	38,031 middle-aged men	20 years	Alcohol: increase from <5.0 to 5.0-29.9 g/day within 4 years	Verified cases of incident type 2 diabetes (N = 1,905)	Hazard ratio	0.75 (0.62, 0.90) ²
2			Alcohol: increase of 7.5 g/day within 4 years	Verified cases of incident type 2 diabetes (N = 1,905)	Hazard ratio	0.78 (0.60, 1.00)
2	697 middle-aged men	4 years	Alcohol: increase of 7.5 g/day within 4 years	Total adiponectin	Mean increment	1.2 µg/mL (0.6, 1.8)
3	35,625 men and women	13 years	Alcohol: men: 5.0-29.9 g/day women: 5.0-14.9 g/day	Verified cases of incident type 2 diabetes (N = 697)	Hazard ratio	0.56 (0.32, 1.00)
Randomized crossover trials						
4				HOMA-IR	Mean difference	-11.9% (-22.1, -1.6)
4				Triglycerides	Mean difference	-8.2% (-15.9 -0.3)
4	36 post-menopausal women	6 weeks	White wine: 25 g alcohol/day	Total adiponectin	Mean difference	10.6% (6.5, 14.7)
4				HMW adiponectin	Mean difference	15.4% (1.4, 29.4)
4				Adipose mRNA adiponectin levels	Mean difference	27.7% (2.8, 58.2)
5				Total adiponectin	Mean difference	8.6 (3.0, 14.2)
5				IL-1 receptor agonist	Mean difference	-13.8 (-30.7, 3.1)
5	24 young men	4 weeks	Vodka: 30 g alcohol/day	IL-18	Mean difference	-4.0% (-9.3, 1.3)
5				Immune response	Network	³
5				PPARα	Network	³

6	24 pre-menopausal women	3 weeks	Beer: 26 g alcohol/day	Total adiponectin	Mean difference	8.2% (2.5, 13.9)
6				HMW adiponectin	Mean difference	8.2% (1.0, 15.4)
6				HMW / total adiponectin	Mean difference	-0.1% (-3.8, 3.6)
7				HDL-cholesterol	Mean difference	10.3% (5.6, 15.0)
7	22 young men	4 weeks	Vodka: 30 g alcohol/day	Apolipoprotein A-I	Mean difference	11.9% (6.1, 17.7)
7				AUC _{6 hours} TNF- α	Mean difference	14.5% (-8.0, 37.0) ⁴
7				AUC _{6 hours} total WBC	Mean difference	1.9% (-5.3, 9.2) ⁴
8	22 post-menopausal women	6 minutes	White wine: 25 g of alcohol	AUC _{30 min} free fatty acids	Mean difference	-20.3% (-30.3, -10.4) ⁵

AUC, Area under the curve; CI, Confidence interval; HDL, High-density lipoprotein; HOMA-IR, Homeostasis model assessment of insulin resistance; HMW, High-molecular weight; PPAR α , Peroxisome proliferator-activated receptor alpha; TNF- α , Tumor necrosis factor alpha; WBC, white blood cells.

¹ Alcohol consumption condition vs. no alcohol consumption condition.

² vs. alcohol consumption ≤ 5.0 g/day.

³ Networks based on genes that were significantly different expressed between treatments.

⁴ After an *in vivo* endotoxin bolus (0.06 ng/kg body weight).

⁵ After modified sham-feeding of wine compared with water.

2 diabetes as shown in the meta-analysis by Baliunas and colleagues in which they found lower risk reductions when only lifelong abstainers were included in the reference group (4). Our results, however, are not likely to be influenced by such bias. In the Dutch cohort, we used lifelong abstainers as referent. In this particular study, former drinker and thus potential ‘sick-quitters’ were included in the light drinking category. In parts of the analyses of changes in alcohol consumption we used current stable drinkers as a referent category instead of current non drinkers thereby bypassing this potential bias. Furthermore, in sensitivity analyses we removed current nondrinkers (which include people who might have given up drinking for health reasons) with minimal effect on the risk estimates. Therefore it is unlikely that our results are biased by the effect of sick-quitters.

Confounding

The results presented could be in part influenced by differences in (lifestyle) factors other than alcohol consumption that are associated with both exposure and disease but that do not lie in the causal pathway between the exposure and the outcome, i.e. confounding. In general, moderate drinkers tend to be leaner and eat better, exercise more and have a higher social economic status (5;6). However, drinkers are also more likely to be smokers and smoking is a risk factor for diabetes. Thus, it is overly simplistic to suggest that alcohol consumption is completely intertwined with a healthy lifestyle. In the two cohort studies presented, we indeed noted that moderate drinkers were likely to exercise and smoke more and were less likely to have a family history of diabetes. Also among females, but not among males, moderate drinkers tended to be leaner than abstainers. Finally, moderate drinkers were higher educated in the Dutch cohort. However, in the U.S. cohort all men were health professionals (e.g. dentists, optometrists, osteopaths, pharmacists, podiatrists, and veterinarians) and thus highly educated, which substantially reduced the potential confounding due to social economic status in this group. We therefore adjusted for a large number of important risk factors of type 2 diabetes such as age, body mass index (BMI), physical activity, smoking, family history of diabetes and several nutritional factors in both studies.

In the observational study on alcohol consumption and lifestyle factors among Dutch men and women, we examined the association of alcohol intake with risk of type 2 diabetes in strata of separate lifestyle behaviors based on body mass index, physical activity level, smoking, and adherence to a healthy diet and in strata of combined low-risk lifestyle behaviors. In the separate strata of each of the four individual lifestyle behaviors and in the strata of joint lifestyle behaviors, moderate alcohol consumers had consistently lower risk of type 2 diabetes compared to abstainers. This makes a compelling case that the inverse association between moderate alcohol and diabetes risk is not confounded by other lifestyle behaviors.

In the study on changes in alcohol consumption, we accounted for changes in several potential confounding factors. First we controlled for changes in hypertension, hypercholesterolemia, and CVD by including terms for these factors at the beginning and

end of the period of change in alcohol consumption in the model. Thus, our results accounted for changes in these risk factors and indirectly for risk factors strongly related to them. In sensitivity analyses, we accounted for the differences in BMI that may have occurred during the four-year periods between assessments of alcohol consumption by including BMI pre- and post- the period of change in alcohol consumption. This did not materially alter the results. We used the exact same approach to assess the effect of changes in physical activity, another important diabetes risk factor. Again, this yielded similar results. There was not sufficient variation in smoking status in this cohort, as the prevalence of current smokers was ~10%, to adjust for change in smoking status. Hence change in smoking was unlikely to be an important confounder. Our approach for dietary risk factors also inherently accounted for change in intake over time. For each dietary factor, we calculated a cumulative average over the period of follow-up, thus including both time points in the models. Because individual dietary factors are substantially less strongly associated with diabetes risk than are BMI and physical activity, it is unlikely that any remaining uncontrolled confounding that is not already accounted for drove the observed findings. Nevertheless, we cannot completely rule out the possibility of residual confounding. Although some residual confounding may attenuate the observed association, it is unlikely that a confounder of this magnitude would have remained undetected.

Assessment of alcohol intake

In the population based studies, we relied on self-reported alcohol consumption which was assessed by a food frequency questionnaire (FFQ). It is known that self-reported alcohol intake is generally being under-reported (7;8). Despite this and obvious other limitations, the FFQ is accepted as the standard tool for dietary assessment in large studies on diet and chronic disease. The FFQs used in our studies were validated against 24-hour recalls showing high correlations of over 0.7 (9-11). To further validate the self-reported alcohol intake, we also determined the relation between alcohol intake and HDL-cholesterol in a 6.5% sub-cohort of Dutch men and women. In line with previous observations, this analysis showed a linear association between alcohol intake and HDL-cholesterol concentrations in both men and women (12). This means that for alcohol intake an FFQ may be a reasonably valid method for ranking subjects according to intake rather than to estimate absolute amounts of intake although the actual amount and frequency of alcohol consumed by the subjects in these studies may differ from that reported. However, any misclassification due to the FFQ is unlikely to affect this rank order of alcohol consumption.

Randomized crossover trials

Mainly due to the limitations of observational studies, such as uncontrolled or residual confounding, findings from randomized trials are generally believed to provide the highest level of evidence. However, conducting large-scale, long-term, randomized controlled trials with hard endpoints such as type diabetes may be impossible due to obstacles in costs,

logistics and ethical issues. Strictly controlled short-term alcohol feeding studies on intermediates of type 2 diabetes are considered to provide an alternative strategy.

Design of the trials

All intervention studies described in this thesis used a randomized, crossover design. The term ‘randomized’ means that the subjects are randomly assigned to a sequence of treatments (e.g. alcohol vs. non-alcohol or vice versa) and ‘crossover’ means that all subjects participated in the different test conditions. This allowed for within subject comparison of effects, thereby controlling many unpredictable individual variables possibly related to outcome measures and increasing statistical power to detect significant differences. A possible issue with this design is potential ‘carryover’ effects between treatments, which could confound the estimates of the treatment effects. In the statistical models, we checked whether statistical significant carryover effects occurred but this was not the case. Therefore, the presented results are unlikely to be biased by carryover effects.

Dose and type of study substance

The amount of alcohol, which was provided in the form of white wine, beer or vodka to participants in the randomized trials, averaged around 25 g/day for women and 30 g/day for men. This amount equals 2.5 to 3 Dutch units or around 2 U.S. units and exceeds current recommendations. However, these guidelines are directed at prevention of all chronic diseases rather than on a maximum risk reduction of type 2 diabetes. Moreover, the amount used in the experimental trials represented the nadir of the association between alcohol consumption and type 2 diabetes based on several meta-analyses (4;13;14). In concert, daily alcohol administration of 30 g of alcohol/day for eight weeks improved insulin sensitivity among postmenopausal women compared with 15 g or 0 g of alcohol per day (15). Furthermore, our clinical intervention studies aimed to provide an underlying mechanism for the observed association and are not meant to directly translate in a public health message. Finally, since the interventions comprised a relatively short period of time, such an elevated dose ensured sufficient contrast between treatments to find significant differences in relevant outcome parameters.

We used commercially available alcoholic beverages and most of the times their most comparable control as study substances for the trial in order to mimic regular alcohol consumption. Nonetheless differences between study substances exists. However, both observational studies and short-term controlled trials comparing beer, wine and spirits found that the observed effects on type 2 diabetes risk or mediating markers such as adiponectin were independent from beverage type and thus due to the alcohol itself. Nevertheless we cannot rule out the fact that certain beverage-specific constituents other than alcohol (e.g. polyphenols in wine, juice or B vitamins in beer) may have affected specific anti-inflammatory processes or insulin sensitivity despite the fact that we selected comparable non alcoholic beverages as counterparts in most of the studies (e.g. white grape

juice vs. white wine and non-alcoholic beer vs. beer). Taken together, we do not think that choice of beverage has materially influenced our results to a large extent.

Study population of the trials:

Participants in the studies consisted of postmenopausal women and younger women and men who consumed alcohol on a regular basis. Therefore, the results may not readily be generalized to middle-aged men or abstainers. However, previous intervention trials of our group have shown that e.g. adiponectin levels are also increased after alcohol consumption in middle-aged men (16;17). Moreover, even among initial abstainers, increases in alcohol consumption were associated with an increment in adiponectin levels, as described in chapter 2. It is thus unlikely that, the findings, especially concerning the increased adiponectin, are limited to the investigated groups of subjects.

Compliance to treatments:

In any intervention study compliance to study treatments is key to obtain valid results. First compliance was monitored by weekly measurement of urinary ethyl glucuronide, a direct phase II metabolite of ethanol formed by action of UDP-glucuronosyl transferase. Ethyl glucuronide has been reported to be a superior marker with 100% sensitivity as a biomarker of recent drinking (18) and persists in the urine up to ~75 to 85 h after last intake. Additional measures of compliance were increase of serum HDL-cholesterol, daily questionnaires, and return of empty bottles. All of these measures suggested a high compliance to the study procedures.

Sample sizes and duration of the trials

The number of subjects in study, in addition to dose and intervention length, is crucial for statistical power to detect relevant differences. Most of the samples sizes were based on previous research either with moderate alcohol consumption or a comparable intervention, which enabled us to estimate realistic detectable differences in outcome measures between treatments. Furthermore, we also had a low drop-out rate during the studies which added to the retention of sufficient statistical power.

Outcome measures

Adiponectin and markers of inflammation and insulin sensitivity in blood were the main parameters measured in all interventions. These measures are thought to be important intermediates in the development of type 2 diabetes. Blood samples were obtained from the antecubital vein of the forearm and collected in tubes. Samples were centrifuged for 15 minutes at 2.000 g at 4°C, within 15 - 30 minutes after collection and stored at <-70°C. Samples were determined using validated assays according to the manufacturers' specifications. Intra-assay and inter-assay variation usually were below 5%.

Besides circulating markers we also measured gene expression levels in various tissues by means of RT-PCR or microarray analysis. RNA quantity and quality were

verified and no serious deviations were observed. Labeling and hybridization of RNA for each tissue was conducted by the same person to minimize variation.

Finally, we did not measure insulin sensitivity according to the golden standard: the euglycemic, hyperinsulinemic clamp technique. Instead, we used an indirect measure based on fasting levels of glucose and insulin (HOMA-IR) which correlates with the clamp technique (19).

Comparison with other studies and interpretation of the findings

Prospective cohort studies

In concert with previous prospective population-based studies from different geographical regions (20-52), we observed an inverse association between moderate alcohol consumption and risk of type 2 diabetes among U.S. men and Dutch men and women. In contrast we did not observe an increased risk of diabetes in heavier consumption categories as was expected based on the reported U-shaped association. In the Dutch cohort, heavier alcohol intake was defined as any intake above current guidelines. This may have obscured a potential U-shaped curve since alcohol intake levels up to about 50 g of alcohol/day (4;14) are still protective against diabetes. In the U.S. cohort, we found a linearly inverse association between alcohol and diabetes, despite a more sophisticated categorization of alcohol intake which should have enabled us to detect a U-shaped curve.

Changes in risk of type 2 diabetes were evident within a 4-year period following changes in alcohol intake (**chapter 2**). This is in line with observations of change in alcohol intake and subsequent change in risk of cardiovascular diseases (53-55) and mortality (56), although some inconsistency exists (57;58). Our findings suggest that the effect of alcohol consumption on diabetes risk has a relatively short latency time but may also be transient and reversible. Furthermore, the temporality of the relation between alcohol and diabetes risk may imply a causal association between alcohol intake and diabetes risk.

Consistent with previous findings, we found that the association between alcohol consumption and type 2 diabetes appeared to be independent of other single (25;32;38) or joint (48;59) lifestyle factors related to diabetes risk. Our results (**chapter 3**) reflect similar additional protection from alcohol consumption for type 2 diabetes among otherwise healthy subjects as was previously shown for heart disease by Mukamal and colleagues (60). The results of these two studies together indicate that the relation is not likely to be explained by a healthier lifestyle of moderate drinkers in general. Also, it shows that the common suggestion by some that alcohol should be avoided since the same protection against these diseases can be caused by other lifestyle habits may not be completely valid.

For the interpretation of observational data on alcohol consumption, one should bear in mind that such results are based on studies where individuals report their average intake as drinks per day, month or year which is in turn transformed into average intake/day. It is unlikely that most individuals drink every day. Yet, current guidelines do not reflect this in the definition for moderation and are based on this exact threshold of “1 drink per day for women or two drinks per day for men” instead of a general “average” metric over the course of a week or month instead. One can thus argue whether these observational data are optimal to derive absolute values for intake recommendations, as misjudgments with respect to the health effects of alcohol intake might occur.

Randomized crossover trial

Whether alcohol consumption improves markers of insulin sensitivity has been examined in several randomized trials of which an overview is given in **chapter 1** (Table 1.1). The only two studies that did report significant changes in markers of insulin sensitivity had intervention periods of 8 weeks and were conducted with older and less insulin-sensitive but non-diabetic men and women (61) or postmenopausal women (15). In line with the latter (15), we also found (**chapter 4**) lowered fasting serum insulin and triglyceride levels and an improvement in insulin sensitivity as estimated by the homeostasis model assessment of insulin resistance (HOMA-IR) among postmenopausal.

However, the majority of the published studies did not find an improvement in (markers of) insulin sensitivity attributed to moderate alcohol consumption. These studies generally used younger adults (age <50 y) (62-67) or had a relatively short treatment period of alcohol consumption (all ≤ 4 weeks) (16;17;68). Hence, it was no surprise that we did not find changes in fasting serum insulin levels or other markers of insulin sensitivity in relatively young and insulin sensitive adults after 3 or 4 weeks of alcohol consumption compared with abstention (**chapters 5 and 6**). However, it should be noted that a myriad of other differences in designs between studies were present (e.g. difference in measurement of insulin sensitivity, dose of alcohol, beverage type, frequency of consumption, amount of participants). Therefore, it cannot be ruled out that experimental factors other than study population and duration are not important.

In line with previous research, (16;17;66;69) moderate alcohol consumption increased circulating adiponectin levels (**chapters 4-6**). This effect was irrespective of beverage type suggesting it is the alcohol that provokes the effect. We could not confirm that alcohol particularly increased the high-molecular weight form of adiponectin, as previously shown (66). The increases in adiponectin after moderate alcohol consumption in the different studies were about 10%, which is a stronger effect than what can be obtained by similar changes in other macronutrients (i.e., a 10% shift in calories) (70;71).

Several lines of evidence suggest an inhibition of the pluripotent transcription factor nuclear factor kappa B (NF- κ B) in white blood cells after acute alcohol consumption (72;73). In concord, we observed in **chapter 5** down-regulation of the NF- κ B and several physiologically related genes under its control after prolonged moderate alcohol consumption. Activation of the transcription factor NF- κ B is a critical step in monocytes inflammatory cytokine production after exposure to bacterial stimuli such as LPS. Yet we did not observe an attenuated cytokine response after a low dose endotoxin administration in men after four weeks of alcohol consumption despite an improved lipoprotein profile (**chapter 7**).

To the best of our knowledge, the preprandial effects of alcohol after mere oral exposure as described in **chapter 8** have never been studied. However several studies on moderate alcohol consumption with a meal have shown several beneficial acute postprandial effects on glycemia (74;75) but also on free fatty acid (FFA) levels and insulin sensitivity (76). The temporary but substantial drop in FFA levels observed in the preprandial study could acutely improve insulin sensitivity and may in turn accumulatively over time result in a lower risk of type 2 diabetes.

Public health implications: to drink or not to drink?

The results in this thesis again underline that moderate alcohol consumption lowers the risk of type 2 diabetes. In addition, the results showed that the association is affected by change in consumption over time and that the association is not confounded by lifestyle behaviors that influence diabetes risk.

Although our results might be seen as suggesting that some individuals should consider adopting regular and moderate intake of alcohol, our findings, even if proven to be causal, are limited to a single outcome of type 2 diabetes. Besides reduced diabetes risk there is also compelling evidence that moderate alcohol intake affects several other health outcomes which should be taken into account. An average daily intake of one to two alcoholic beverages is associated with the lowest all-cause mortality (77) and a reduced risk of cardiovascular diseases (78;79) but also with higher risk of breast cancer among women (80) especially with risk of hormone-sensitive breast cancers (81;82). Besides these effects with moderate alcohol consumption, there is evidence for a positive associations between heavy alcohol consumption and risk of unintentional injuries (83), liver cirrhosis (84), some forms of cancer (85), and addiction which should also be taken into consideration. Current guidelines acknowledge this by recommending that if any alcohol is consumed at all, it should be consumed in moderation, and only by adults. Moreover, studies suggest adverse effects at even moderate amounts of alcohol consumption in specific individuals and situations. According to the U.S. National Institute of Alcohol Abuse and Alcoholism (86) and the Netherlands Health Counsel (87) people who should abstain include:

- Children and adolescents.
- Women who are pregnant or planning to become pregnant.
- Individuals with specific medical conditions (e.g. liver disease, pancreatitis).
- Individuals taking medications that can interact with alcohol.
- Individuals who plan to engage in activities that require alertness and skill (such as driving a car).
- Individuals who cannot restrict their drinking to moderate levels or are recovering from alcoholism.

For teetotalers it is undesirable to advise picking up the habit of consuming alcoholic beverages in moderation. Usually they have their motives to abstain (e.g. religion or family history of alcoholism).

Some argue that moderate alcohol intake is only effective in middle-aged and older people to prevent chronic diseases. This seems especially the case for outcomes like cardiovascular diseases and type 2 diabetes that appear to be more malleable over the course of a lifetime than e.g. cancer. Indeed, research in this thesis has shown that adopting moderate alcohol intake later in life could reduce the risk of type 2 diabetes as is the case for risk of cardiovascular diseases (53;56). For younger adults there may be less health benefit from alcohol drinking throughout adulthood because the preventive effect of moderate alcohol consumption on these diseases can only work if there is an actual risk. These risks become evident at about middle age whereas risks associated with the negative consequence of alcohol consumption are prevalent throughout adulthood. On the other hand, although risks of chronic diseases become actual at older age, it has been generated by a series of events in the previous decades. It might therefore, be overly simplistic to ignore the fact that earlier signs of e.g. atherosclerosis (such as fatty streaks) can already be observed during adolescence. Furthermore, there is evidence that alcohol consumption does reduce the risk of type 2 diabetes (38) and cardiovascular diseases (88) among younger adults. Thus, it may be somewhat premature to suggest that only middle-aged and older adults can benefit from light or moderate alcohol consumption. Especially, since a fundamental principle of dietary guidelines is the concept that a healthy diet starting from a young age can mitigate such processes and hence reduce the risk of chronic diseases in later life.

Guidelines for a healthy lifestyle generally do not recommend moderate alcohol consumption because of the risks associated with heavy alcohol consumption. Usually other lifestyle features are emphasized. A drawback with such an approach is that healthy lifestyle behaviors are often inter-correlated and may be most effective when present in combination and generally should not be considered mutually exclusive. Possibly, light to moderate alcohol consumption could be an additional part of such a healthy lifestyle.

However caution is warranted and tailored advice on alcohol consumption is essential. Heavy or excessive drinking should always be discouraged, whereas moderate alcohol consumption could be regarded as a complement, rather than an alternative, to other low-risk lifestyle habits. Guidelines and personal advice on alcohol consumption should consider the full range of benefits and risks to the individual including the consistent harms to the individual and society of drinking that exceeds recommended limits. A discussion with a health care provider is strongly advised for all people considering a change in their drinking habits.

Future research recommendations

Observational studies

Genetic predisposition

It is recommended to pay more attention to the genetic variation in genes coding for enzymes involved in alcohol metabolism since large-scale clinical trials with hard endpoints such as type 2 diabetes are not feasible in this field. An individual's genetic composition does not change over time and is less likely to be associated with confounding factors. Therefore, research on the effect modifying potential of genetic variants, for example ADH and ALDH, which favor a casual relationship between alcohol intake and disease, may provide more insight. Along this line, a more sophisticated approach could be to compose a gene score based on some *a priori* chosen gene variants that predicts alcohol consumption. If this score is associated with both alcohol consumption and diabetes risk in another, independent cohort this would provide compelling evidence for a causal relationship between alcohol consumption and type 2 diabetes.

Biomarkers mediating of the association between alcohol consumption and diabetes

Although the association between moderate alcohol consumption and decreased risk of type 2 diabetes has been reported consistently, limited observational data are available on potential underlying mechanisms. For alcohol consumption and cardiovascular disease this relation may be mediated through HDL-cholesterol and non HDL-cholesterol factors, such as insulin sensitivity, inflammatory markers, lipid and haemostatic factors (12;89-91). For type 2 diabetes, adiponectin appeared to explain about 25% of the relation between alcohol and diabetes risk whereas circulating biomarkers of inflammation, endothelial dysfunction, and fasting insulin did not explain a substantial part of this association (92). However, this specific study solely consisted of middle-aged women, had a case control design, and was performed with a limited number of potential mediators. For example, triglyceride concentrations were not included in this model despite being an independent risk factor for diabetes (93). Compared with postmenopausal non-drinkers, postmenopausal drinkers have

lower triglyceride levels whereas male drinkers have higher triglyceride levels compared with male abstainers (94).

Nevertheless, studies like these may help to clarify the possible mechanisms underlying the observed association between moderate alcohol intake and diabetes. Therefore, future research should confirm the mediating effect of adiponectin, not only in women but also in men. Furthermore, additional potential mediators such as triglycerides and inflammatory markers such as TNF- α should be taken into account in these analyses besides the more traditional glycemic and inflammatory markers.

Drinking pattern

Measures of overall volume of alcohol consumption obscure the relative contributions of drinking frequency (how often alcohol is consumed), drinking quantity (how much alcohol is typically consumed on those days), and their individual contributions to diabetes risk have not been thoroughly investigated. Therefore, the effect of drinking pattern should be examined further in future studies.

Randomized trials

The physiological mechanisms that could account for the risk reduction in type 2 diabetes associated with moderate alcohol consumption are not completely understood. However, several theories have been proposed such as an improved insulin sensitivity, increased adiponectin levels, lowered inflammatory status and improved glycemic control.

In order to provide more definite answers, future randomized trials with prolonged moderate alcohol consumption might consider using:

- subjects with slightly impaired insulin sensitivity of increased risk of type 2 diabetes (and thus more likely to benefit from alcohol consumption),
- sufficiently long treatment periods (preferably over six weeks of moderate alcohol consumption to find improvements in insulin sensitivity),
- other or more convincing intermediates of type 2 diabetes e.g.
 - Profiling of other tissues besides blood that might be relevant tissues in the etiology of type 2 diabetes such as (visceral) adipose or liver tissue.
 - β -cell function and secretion of related incretin hormones such as glucagon-like peptide-1 (GLP-1) and gastric inhibitory peptide / glucose-dependent insulinotropic polypeptide (GIP).
 - Challenges of homeostasis (oral glucose tolerance test or euglycemic clamp to challenge insulin sensitivity /glycemic control or an endotoxin challenge to characterize the inflammatory or immune response.
- an integrated approach of metabolic, protein, and gene profiling of different tissues for the discovery of potential new biomarkers.

Finally, investigating the more acute effects associated with moderate alcohol consumption on e.g. preprandial (cephalic phase) or postprandial responses and its subsequent consequences could help to elucidate the relation between alcohol and insulin sensitivity or glycemia which in turn could accumulatively affect diabetes risk.

Concluding remarks

In conclusion, research presented in this thesis confirmed the reduced risk of type 2 diabetes associated with moderate alcohol consumption in two separate populations. Furthermore, it extended the association by showing the temporality of the association, and by expanding this relation to subjects already at lower risk of type 2 diabetes. Finally, it provided several plausible physiological mechanisms that may account for the lower risk of type 2 diabetes.

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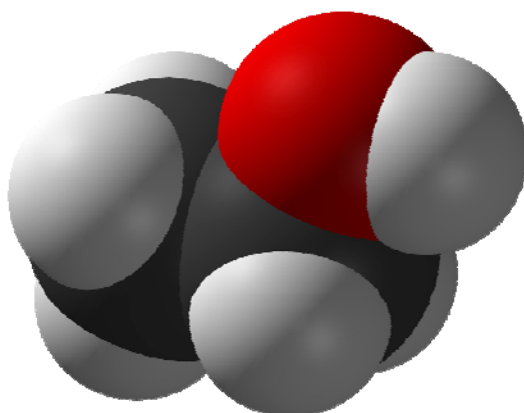
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Summary



Moderate alcohol consumption has been associated with a reduced risk of type 2 diabetes compared with abstinence. Despite overwhelming evidence for this relation from observational studies and to some extent from clinical trials on intermediate endpoints, several questions concerning this relation remain. This thesis thus aimed to substantiate the evidence of the inverse association between moderate alcohol consumption and type 2 diabetes in different populations in observational studies and to provide underlying physiological mechanisms in clinical intervention studies.

Observational studies

Changes in alcohol consumption over time are associated with subsequent changes in risk of cardiovascular diseases and mortality but whether changes in alcohol consumption influence future diabetes risk was uncertain. Thus in **chapter 2** we investigated among 38,031 middle-aged U.S. men if four-year changes in alcohol consumption affected subsequent type 2 diabetes risk. Among initially rare and light drinkers, moderate increases in alcohol consumption over a four-year time period were associated with a lower risk of type 2 diabetes. However, individuals already consuming alcohol in moderation did not receive further benefit from increased consumption. The results suggest that the effects of alcohol consumption on type 2 diabetes risk have a relatively short latency time but are also transient and reversible.

Whether the lower risk of type 2 diabetes among subjects who consume alcohol is due to the alcohol drinking itself or whether, to some extent, it is due to other healthy lifestyle factors of drinkers is a continuing question. In **chapter 3**, we determined if alcohol drinking, in addition to having optimal weight, being physically active, nonsmoking, and consuming a healthy diet, had an effect on the risk of developing type 2 diabetes among 35,625 Dutch men and women. The results showed that even among subjects who are at lower risk of developing diabetes on the basis of other lifestyle characteristics, alcohol consumption provided considerably further decrease of about 40% in the risk of type 2 diabetes.

Intervention studies

Although moderate alcohol consumption has consistently been associated with a decreased risk of type 2 diabetes compared with abstaining it remains uncertain whether it improves insulin sensitivity. In **chapter 4**, we describe the results of a randomized, crossover trial among thirty-six apparently healthy postmenopausal women who received white wine (~25 g alcohol/day) or white grape juice daily during dinner for six weeks. Moderate alcohol consumption for six weeks improved insulin sensitivity by about 11% and adiponectin levels by about 12% in postmenopausal women. Furthermore, these data suggested a transcriptional mechanism leading to the alcohol-induced increase in adiponectin plasma levels.

Moderate alcohol consumption has several acute effects on gene expression of white blood cells but longer term effects on gene expression are largely unknown. Thus in **chapter 5** we examined with large-scale profiling of proteins and genes the effects of four weeks of moderate vodka (~30 g alcohol/day) consumption on inflammatory markers and gene expression profiles in blood of twenty-four younger men. This integrated approach of large-scale profiling of proteins and genes revealed that moderate alcohol consumption for four weeks altered gene expression profiles of white blood cells and circulating markers related to inflammation.

Previously, we have shown that moderate alcohol consumption increased levels of adiponectin, a hormone inversely associated with type 2 diabetes risk. However, the rate at which this increase occurs is unclear and if this increase differs for specific forms of adiponectin was also uncertain. In **chapter 6**, we therefore examined the effect of moderate alcohol consumption on the weekly alterations in levels of both total and high-molecular weight (HMW) adiponectin in twenty-four premenopausal women. We observed that moderate beer consumption (~26 g alcohol/day) increased both the high-molecular weight form as the total adiponectin from by about 8% after at least three weeks of consumption compared with alcohol-free beer consumption. Alcohol consumption did not preferentially change the molecular weight distribution of adiponectin in these younger women.

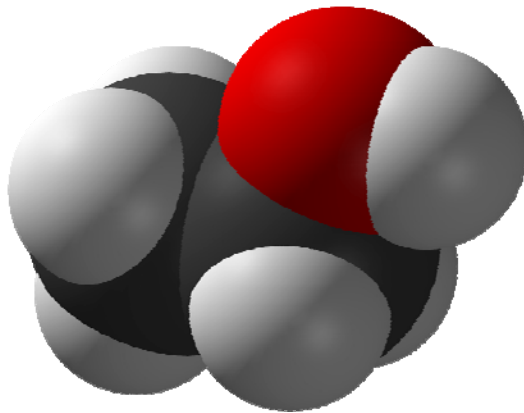
Circulating high-density lipoprotein (HDL)-cholesterol and its associated surface lipids apolipoproteins have been shown to play an important role in the detoxification of a specific endotoxin named LipoPolySaccarides (LPS). Moderate alcohol consumption increases HDL-cholesterol and several apolipoproteins. In **chapter 7**, we aimed to study whether the alcohol-induced increase in lipoproteins attenuated the inflammatory response after a low-dose *in vivo* endotoxin challenge. After four weeks of moderate vodka juice consumption (~30 g alcohol/day) several lipoproteins were increased by approximately 10% compared with four weeks of juice consumption in twenty-two men. However, we did not observe an attenuated inflammatory response over time after a low-dose *in vivo* endotoxin bolus.

Preingestive or cephalic phase responses, triggered by sensory stimulation of macronutrients, influence the organism's endocrine responses and substrate mobilization. However whether oral sensory stimulation with alcohol elicits cephalic phase responses was unknown. In **chapter 8**, we investigated whether short-term (~6 min) oral alcohol exposure, in the form of white wine(~25 g alcohol), provoked cephalic phase responses in eleven normal-weight and eleven overweight subjects. Oral white wine exposure in the oral cavity substantially (~20%) and temporarily (~20 min) decreased serum free fatty acid concentrations compared with water exposure regardless of BMI. This suggests a cephalic phase response of alcohol.

| Summary

The main findings, methodological considerations and interpretation of the findings of the studies described in this thesis are discussed in **chapter 9**. Directions for further research and implications for public health are mentioned

Lay summary in Dutch
(Samenvatting voor niet-ingewijden)



Het is al langere tijd bekend dat er een relatie is tussen *matige alcoholconsumptie** en een lager risico op het krijgen van diabetes mellitus type 2 (kortweg *diabetes type 2**) in vergelijking met geheelonthouding of overmatige alcoholconsumptie. Ondanks veelvuldig bewijs was het nog steeds niet helemaal duidelijk of dit verband daadwerkelijk veroorzaakt wordt door de alcoholconsumptie of door andere factoren, die gepaard gaan met matige alcoholconsumptie. Ook was het nog grotendeels onbekend wat alcohol in het lichaam doet om de verlaging in het risico op diabetes type 2 te kunnen verklaren.

Het doel van dit proefschrift is tweeledig:

- 1) Het bewijs voor de relatie tussen matige alcoholconsumptie en diabetes type 2 verder uit te breiden door middel van **bevolkingsonderzoek** met diabetes type 2 als eindpunt.
- 2) Mogelijk onderliggende mechanismen te onderzoeken, welke het verband vanuit een biologisch oogpunt kunnen verklaren. Dit is gedaan met behulp van **interventiestudies** met belangrijke voorspellers van diabetes type 2 als uitkomst.

* Matige alcoholconsumptie

Matige alcoholconsumptie wordt door richtlijnen omschrijven als maximaal één standaard glas voor vrouwen en maximaal twee standaard glazen per dag voor mannen.

Dagelijks 2 tot 3 glazen alcohol gaat samen met het laagste risico op diabetes type 2. Daarom definiëren we in dit proefschrift deze hoeveelheid als matige alcoholconsumptie.

Algemene inleiding

In **hoofdstuk 1** bespreken we de definitie van matige alcoholconsumptie. Ook geven we in dit inleidende hoofdstuk een omschrijving van diabetes type 2 en het ontstaan ervan. Daarnaast geven we een overzicht van de meest recente uitkomsten uit bevolkingsonderzoek waaruit blijkt dat er een verband is tussen alcoholconsumptie en een verlaging van het risico op diabetes type 2. Verder bespreken we de mogelijk onderliggende mechanismen op basis van eerder uitgevoerde interventiestudies. Tenslotte beschrijven we de doelstellingen en de opbouw van dit proefschrift.

* Diabetes type 2

Diabetes type 2, ofwel ouderdomsuikerziekte, is een ziekte die wordt gekenmerkt door een verhoogd glucose (= suiker) gehalte in het bloed. Bij dit type diabetes produceert het lichaam wel insuline maar is het lichaam minder gevoelig voor insuline geworden.

Bevolkingsonderzoek

Met bevolkingsonderzoek bedoelen we een studieopzet waarbij in een grote groep mensen wordt nagegaan in welke mate een bepaalde ziekte onder de bevolking voorkomt en welke factoren dit mogelijk in de hand werken of juist voorkómen. In dit proefschrift kijken we specifiek naar alcoholconsumptie maar houden daarbij ook rekening met leeftijd, leefstijlfactoren zoals lichaamsgewicht, beweging, voedingspatroon en rookgedrag, en andere belangrijke factoren zoals familiegeschiedenis van diabetes en opleidingsniveau. Statistische analyses van vaak grote databestanden zijn daarbij een belangrijk hulpmiddel.

Effect van verandering in alcohol op diabetes

Een relatief nieuwe methode om na te gaan hoe ‘hard’ het veronderstelde verband tussen matige alcoholconsumptie en diabetes type 2 is, is om de ‘tijdelijkheid’ van dit verband te onderzoeken. Met andere woorden: hoe beïnvloedt een toename of afname in alcoholconsumptie in een bepaalde tijdsperiode het risico op diabetes type 2? In **hoofdstuk 2** hebben we beschreven hoe dit zich gedurende twintig jaar bij 38.031 Amerikaanse mannen van middelbare leeftijd ontwikkelt. Hierbij hebben we specifiek gekeken of veranderingen in de alcoholconsumptie over periodes van vier jaar een effect hadden op het risico op diabetes type 2 in de daaropvolgende vier jaar. Hierbij hebben we ook rekening gehouden met andere relevante veranderingen die mogelijk van invloed zijn, zoals lichaamsgewicht, lichaamsbeweging, rookgedrag en voedingspatroon. Uit het onderzoek bleek dat wanneer personen die slechts incidenteel alcohol dronken hun consumptie van alcoholhoudende drank verhoogden tot een matig niveau, dit leidde tot 10% hogere gehalten van *adiponectine** in het bloed en tot een 22% lagere kans op diabetes type 2. Omgekeerd, wanneer matige drinkers hun alcoholconsumptie verlaagden, nam het risico op diabetes type 2 toe tot een niveau dat vergelijkbaar was met het risico van mensen die aanvankelijk incidenteel alcoholhoudende dranken consumeerden. Bij mensen die bij aanvang van de studie matig dronken en in de loop der tijd hun consumptie verhoogden, bleek er echter geen sprake te zijn van extra bescherming tegen diabetes type 2. Deze resultaten laten zien dat het effect van alcohol op het risico op diabetes type 2 na een relatief korte tijd (vier jaar) zijn beslag krijgt, maar ook dat het effect in een zelfde tijdsperiode teniet gedaan kan worden wanneer de consumptiehoeveelheid verandert.

*** Adiponectine**

Adiponectine is een hormoon dat vrijwel alleen wordt gemaakt en afgegeven door het vetweefsel. Hoge gehalten van dit hormoon in het bloed hangen samen met een hoge insulinegevoeligheid, een lage ontstekingsgraad en een lager risico op diabetes type 2 en hart- en vaatziekten.

Nog effect van alcohol op diabetes bij een gezonde leefstijl?

Diabetes type 2 kan veelal worden voorkomen door een gezonde leefstijl. Belangrijke elementen van een gezonde leefstijl zijn: het voorkomen van overgewicht, voldoende lichaamsbeweging, niet roken en een uitgebalanceerd voedingspatroon. Of bij mensen die er al een gezonde leefstijl op na hielden, matige alcoholconsumptie toch nog een toegevoegd gunstig effect heeft op het risico op diabetes type 2 was nog niet eerder onderzocht. In **hoofdstuk 3** is beschreven hoe we dit bij 35.625 Nederlandse mannen en vrouwen tussen de 20 en 70 jaar gedurende 10 jaar hebben onderzocht. De resultaten lieten zien dat personen die zich hielden aan ten minste drie van de bovengenoemde beschermende leefgewoonten én daarnaast een matige hoeveelheid alcoholhoudende drank consumeren, 40% minder kans hebben op het ontwikkelen van diabetes type 2 dan geheelonthouders met een gezonde leefstijl.

Interventiestudies

Bij interventiestudies wordt aan een relatief kleine groep proefpersonen een bepaalde stof of behandeling gegeven. In de beschreven interventiestudies in dit proefschrift werden alcoholhoudende dranken en alcoholvrije controle dranken aan de proefpersonen gegeven. De interventiestudies duurden 8 tot 12 weken en zijn uitgevoerd volgens een zogenaamde 'gerandomiseerde cross-over' opzet. Dat betekent dat alle proefpersonen beide dranken (alcohol vs. alcoholvrij) hebben gekregen (= cross-over) maar in een willekeurige volgorde (= gerandomiseerd). Mannen dronken bij de avondmaaltijd dagelijks 3 standaard glazen en vrouwen 2,5 glazen. Een standaard glas bevat ongeveer 10 gram pure alcohol, ongeacht het type drank (bier, wijn of gedistilleerd). Aan het einde van beide periodes werd lichaamsmateriaal (bloed, vetweefsel, urine of speeksel) afgenomen om veranderingen in uitkomstmaten te bestuderen.

Veranderingen in insulinegevoeligheid door alcohol

Hoewel uit een groot aantal onderzoeken blijkt dat mensen die een matige hoeveelheid alcohol consumeren een verlaagd risico hebben op diabetes type 2 in vergelijking met geheelonthouders, was het nog onduidelijk of alcoholconsumptie de *insulinegevoeligheid** van het lichaam verhoogt. In **hoofdstuk 4** beschrijven we de resultaten van een klinische interventiestudie onder 36 vrouwen van middelbare leeftijd (na de overgang) die gedurende zes weken witte wijn en wit druivensap hebben gedronken. Matige alcoholconsumptie bleek de insulinegevoeligheid met 11% en het gehalte adiponectine in het bloed met 12% te laten toenemen in vergelijking met zes weken onthouding van alcoholhoudende drank. Bovendien bleek dat de toename van adiponectine in het bloed toe te schrijven was aan een toegenomen aanmaak van adiponectine in het vetweefsel.

*** Insulinegevoeligheid**

Dit betekent de gevoeligheid van weefsels zoals lever en spieren voor insuline. Insuline zorgt ervoor dat glucose in de weefsels komt. Een verlaagde insulinegevoeligheid kan een voorspeller zijn van diabetes type 2.

Effect van alcohol op witte bloedcellen

Matige alcoholconsumptie heeft verschillende korte termijn effecten op de genexpressie (= het tot uitdrukking komen van erfelijke eigenschappen) van witte bloedcellen. Deze cellen spelen een belangrijke rol bij een *ontsteking** en bij de afweer van het lichaam. De effecten van matige alcoholconsumptie op deze witte bloedcellen op een wat langere termijn zijn nog grotendeels onbekend. Om dit te onderzoeken hebben we in **hoofdstuk 5** beschreven hoe we de veranderingen in eiwitten in bloed en in genexpressie van witte bloedcellen hebben onderzocht. Hiervoor hebben 24 jonge mannen vier weken lang dagelijks een mix van jus d'orange met wodka gedronken en vier weken alleen jus d'orange gedronken. De

*** Ontsteking**

Een beschermende reactie van lichaamswefsel op een schadelijke prikkel, vaak gekenmerkt door een rode, warme, pijnlijke zwelling.

veranderingen in zowel eiwitten als genexpressie liet zien dat na de periode van alcoholconsumptie verschillende processen op het gebied van ontsteking en vetmetabolisme waren veranderd. Deze veranderingen gaven inzicht in de manier waarop alcohol mogelijk de kans op diabetes type 2 verlaagt.

Effect van alcohol op het hormoon adiponectine

Eerder onderzoek heeft aangetoond dat matige alcoholconsumptie bij jonge mannen en bij mannen en vrouwen van middelbare leeftijd het adiponectinegehaltes in het bloed verhoogt (zie hoofdstuk 4). Of alcoholconsumptie ook bij jongere vrouwen de adiponectinewaarden in het bloed laat stijgen was nog niet eerder onderzocht. Ook de snelheid waarmee dit gebeurt, was nog onduidelijk. Bovendien was nog onvoldoende bekend of deze stijging na alcoholconsumptie ook optreedt voor de hoogmoleculaire vorm van adiponectine. Aangenomen wordt dat vooral deze vorm de ‘biologisch actieve’ vorm is. Daarom hebben we in **hoofdstuk 6** bij 24 jonge vrouwen de wekelijkse veranderingen in de totale als de hoogmoleculaire vorm van adiponectine gemeten. We zagen dat in vergelijking met alcoholvrij bier, alcoholhoudend bier zowel het gehalte van de totale als de hoogmoleculaire vorm van adiponectine met ongeveer 8% liet toenemen. Deze stijgingen waren pas waarneembaar na minimaal drie weken van matige alcoholconsumptie.

Effect van alcohol op kunstmatige ontstekingsreactie

Matige alcoholconsumptie verhoogt de bloedwaarden van het HDL-cholesterol, ook wel het ‘goede’ cholesterol genoemd. Naast andere functies speelt dit HDL-cholesterol ook een belangrijke rol in de afvoer van schadelijke stoffen. Sommige van die schadelijke stoffen kunnen een ontstekingsreactie kunnen veroorzaken. In **hoofdstuk 7** hebben we daarom onderzocht of de toegenomen waarde van HDL-cholesterol na alcoholconsumptie bij de proefpersonen leidt tot een lagere ontstekingsreactie. Hiervoor hebben 22 mannen gedurende vier weken dagelijks een mix van jus d’orange met wodka of alleen jus d’orange gedronken. Aan het einde van beide periodes werd bij de proefpersonen een milde ontstekingsreactie opgewekt door het toedienen van een schadelijke stof. Hoewel het HDL-cholesterol na vier weken matige alcoholconsumptie 10% hoger was dan na de vier weken zonder alcoholconsumptie, bleek er geen verschil te zijn in de ontstekingsreactie na beide periodes.

Effect van proeven van alcohol op vrije vetzuren

Het proeven van voedsel prikkelt bepaalde zintuigen in de mond. Deze prikkel bereidt het lichaam voor op het voedsel dat doorgeslikt gaat worden. In **hoofdstuk 8** hebben we bij 22 vrouwen van middelbare leeftijd (na de overgang) onderzocht of het proeven van witte wijn gedurende 6 minuten zonder het door te slikken ook een dergelijke reactie opwekt. Na het proeven was de hoeveelheid vrije vetzuren (= een bron van kant-en-klare energie) in het bloed van de wijnproevers 20% lager dan in het bloed van de waterproevers. De daling in vrije vetzuren door het proeven van de wijn was tijdelijk (20 minuten). Mogelijk leidt de

daling van vrije vetzuren in het bloed ten gevolge van het proeven van alcohol tot een tijdelijke verhoging van de insulinegevoeligheid.

Algemene discussie

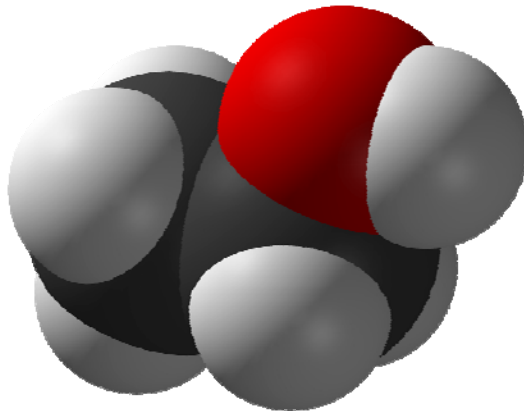
Tot slot wordt in **hoofdstuk 9** een kort overzicht gegeven van de belangrijkste bevindingen van dit proefschrift. Ook worden de voor- en nadelen van de gebruikte onderzoeksmethoden besproken en worden de gevonden resultaten vergeleken met eerdere bevindingen in de wetenschappelijke literatuur. Vervolgens wordt de relevantie van de uitkomsten voor de volksgezondheid beschreven. Tenslotte, worden suggesties gedaan voor verder onderzoek op het gebied van matige alcoholconsumptie en diabetes type 2.

Kortom, dit proefschrift heeft het verband tussen matige alcoholconsumptie en een lager risico op diabetes type 2 uitgebreid door in bevolkinsonderzoek aan te tonen dat:

- het verband ook geldt voor mensen die door hun leefstijl al een verlaagde kans op diabetes type 2 hebben
- een toename in alcoholconsumptie tot een matig niveau leidt tot een daaropvolgende verlaging in het risico op diabetes type 2.

De toegenomen adiponectinegehalten en insulinegevoeligheid en de veranderingen in ontstekingsprocessen na matige alcoholconsumptie in de interventiestudies kunnen de verlaging in risico op diabetes type 2 mogelijk verklaren.

Acknowledgement (Dankwoord)



This is it! De uitkomsten van vier jaar wetenschappelijk onderzoek gebundeld in één boek. Meer dan 750.000 (driekwart miljoen!) persoonsjaren en gegevens van ruim 90.000 mensen uit bevolkingsonderzoeken in Nederland en Amerika, maar ook een slordige 546 liter witte wijn, 70 liter wodka, 665 liter (alcoholvrij)bier, 378 liter witte druivensap, 275 liter jus d'orange en 168 liter mineraalwater plus ongeveer 40 liter bloed, 6,4 liter urine, 36 gram vetweefsel en 70 milliliter speeksel van meer dan 108 verschillende proefpersonen uit vier klinische interventiestudies zijn nodig geweest voor dit resultaat. Het is dan eigenlijk overbodig om te zeggen dat ik al dit werk nooit alleen heb kunnen doen. Ik ben dan ook héél véél dank verschuldigd aan alle mensen die mij, op wat voor manier dan ook, hebben geholpen of bijgestaan in de afgelopen vier jaar.

Ik wil beginnen met mijn copromotor en dagelijkse begeleider bij TNO Kwaliteit van Leven: Dr. Henk F.J. Hendriks (of mag ik na al die jaren wetenschappelijk onderzoek over de gezondheidseffecten van matige alcoholconsumptie spreken over Dr. *Hendrinks?*). Beste Henk, dank voor je vertrouwen in mij en dank voor de ruimte die je me hebt gegeven. Ik heb heel veel geleerd van je tomeloze enthousiasme, je kritische opmerkingen (met af en toe een gefronste wenkbrauw) en je nieuwe theorieën en invalshoeken. Naarmate de jaren verstreken, heb jij het steeds drukker gekregen. Toch had je altijd tijd voor me als dat nodig was. Dit heb ik erg gewaardeerd.

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Dr. Eric B. Rimm. Dear Eric, thank you for giving me the opportunity to work with you, your group and the Department of Nutrition of the HSPH. I appreciate it that you

will come to the Netherlands and participate in my dissertation despite your daughter's 18th Birthday.

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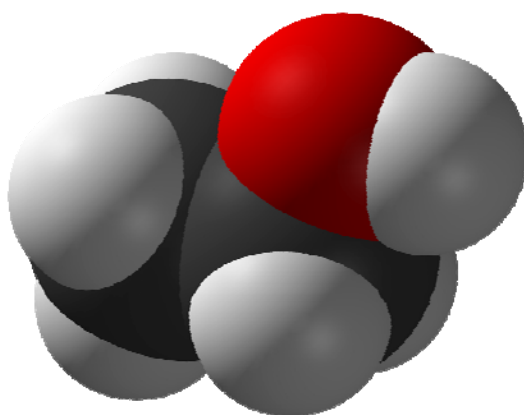
Ik wil nog enkele mensen bedanken die ik in de loop der tijd heb leren kennen in Wageningen. Te beginnen met mijn sportkameraden van het eerste uur: Jochem Sloothaak en Erik van der Galiën. Dank voor de afleiding aan de eettafel, in de gym of op de fiets gedurende al die jaren. Dennis Reinieren, ook jij behoort hier inmiddels toe. Pieter Blok, ‘Vinnie’, na al die fitnessavonden ben je ondertussen ook een ‘voedingsman’ geworden ☺. Echte voedingsmannen en trainingsmaten Frans, Jorn en Jasper: ook jullie bedankt voor het gezelschap bij de schranspartijen en bij de gewichten van de fitness. Mijn ‘chicas’ Wendy Wolters en Yvonne Zebregs, Marieke, Magda en de andere exleden van het Di-et-Tri bestuur en afdelingsgenoten van 14B: ook jullie wil ik bedanken voor het luisterende oor.

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About the author



Curriculum Vitae

Michel Marinus Joosten was born on April 14, 1982 in Rotterdam, the Netherlands. After completing secondary school at the 'St. Montfort College' in Rotterdam he started his bachelor program in 'Nutrition and Health' at the Wageningen University. After having received his B.Sc. degree in 2004, he enrolled in the Master program in 'Nutrition and Health' with a specialization in 'Dietary Behavior and Disease Prevention'. Within this program he performed his M.Sc. thesis in rural North Vietnam on the acceptance and preference of iron-fortified noodles among anemic school children and his internship at the R&D department of the confectionary site of Mars in Veghel, the Netherlands to develop and validate a protocol for satiety testing of snack foods.

In December 2006, Michel was appointed as a Ph.D. fellow to the division of Human Nutrition at the Wageningen University to perform research on alcohol consumption and risk of type 2 diabetes. He was detached at TNO (a Dutch acronym for applied scientific knowledge) Quality of Life in Zeist to design, conduct, analyze, and report data of several human intervention studies on moderate alcohol consumption and on probiotics. He was involved in epidemiological analyses on alcohol consumption and risk of type 2 diabetes in two large prospective cohort studies. During his period as a Ph.D. candidate, he conducted observational research at the Harvard School of Public Health (HSPH) in Boston, MA, U.S.A. during a 5-month stay in close collaboration with Dr. Eric B. Rimm and Dr. Kenneth J. Mukamal. He won a New Investigator Travel Award in 2009 for attending the annual meeting of the Society for the Study of Ingestive Behavior (SSIB) and he was selected for the European Nutritional Leadership Program (ENLP) in 2010. Part of this thesis was nominated for the '*Foppe ten Hoor*' award in 2010, the annual award of the Netherlands Organization for Scientific Research (NWO) for young researchers in the field of scientific nutrition research.



List of publications

Publications in peer-reviewed journals

Joosten MM, de Graaf C, Rietman A, Witkamp RF, Hendriks HFJ. Short-term oral exposure to white wine transiently lowers serum free fatty acids. *Appetite*. 2010; 55:124-9.

Joosten MM, Balvers MGJ, Verhoeckx KC, Hendriks HFJ, Witkamp RF. Plasma anandamide and other *N*-acylethanolamines are correlated with their corresponding free fatty acid levels under both fasting and non-fasting conditions in women. *Nutrition & Metabolism*. 2010; 7:49.

Joosten MM, Grobbee DE, van der A DL, Verschuren WM, Hendriks HFJ, Beulens JWJ. Combined effect of alcohol consumption and lifestyle behaviors on risk of type 2 diabetes. *American Journal of Clinical Nutrition*. 2010; 91:1777-83.

Joosten MM, Beulens JWJ, Kersten S, Hendriks HFJ. Moderate alcohol consumption increases insulin sensitivity and *ADIPOQ* expression in postmenopausal women: a randomised, crossover trial. *Diabetologia*. 2008; 51:1375-81.

Le TH, Joosten MM, van der Bijl J, Brouwer ID, de Graaf C, Kok FJ. The effect of NaFeEDTA on sensory perception and long-term acceptance of instant noodles by Vietnamese schoolchildren. *Food Quality and Preference*. 2007; 18:619–26.

Accepted paper

Joosten MM, Chiuve SE, Mukamal KJ, Hu FB, Hendriks HFJ, Rimm EB. Changes in alcohol consumption and subsequent risk of type 2 diabetes in men. *Diabetes*, 2011.

Submitted papers

Joosten MM, Witkamp RF, Hendriks HFJ. Alterations in total and high-molecular weight adiponectin after moderate alcohol consumption in premenopausal women.

Joosten MM, Boetje M, Witkamp RF, Hendriks HFJ. Alcohol-induced increase in lipoproteins does not alter inflammatory response after low-dose *in vivo* endotoxin challenge in men.

Joosten MM, van Erk MJ, Pellis L, Witkamp RF, Hendriks HFJ. Moderate alcohol consumption alters leukocyte gene expression profiles and proteins related to immune response and lipid metabolism in men.

Other publication

Joosten MM and Hendriks HFJ. Gezondheidsaspecten van matige alcoholconsumptie Past alcohol in een gezond voedingspatroon? Nederlands Tijdschrift voor Voeding & Diëtetiek. 2010; 65:7-10. (in Dutch).

Abstracts in scientific journals

Joosten MM, Witkamp RF, Hendriks HFJ. Postprandial peripheral *N*-acylethanolamides are associated with free fatty acids but not with sensations of appetite and well-being in young normal-weight women. *Appetite*. 2009; 52:840 (Poster presentation).

Joosten MM, De Graaf K, Witkamp RF, Hendriks HFJ. Oral sensory stimulation with alcohol lowers serum free fatty acids in women. *Appetite*. 2009; 52:840 (Oral presentation).

Joosten MM, De Graaf K, Witkamp RF, Hendriks HFJ. Oral sensory stimulation with alcohol suppresses free fatty acids in women. *European Journal of Clinical Nutrition*. 2009; 63 S3:11 (Poster presentation).

Overview of completed training activities

Description	Organizer, location	Year
Discipline specific activities		
Courses and workshops		
Analytical Epidemiology	Division of Human Nutrition, WUR	2007
5 th European NutriGenomics Organization (NuGO) hands-on microarray data analysis course	NuGO, Maastricht (NL)	2009
Conferences and meetings		
Annual meetings NWO	NWO, Deurne (NL)	2007-2010
European Congress on Obesity (ECO)	ECO, Budapest (H)	2007
European Association for the study of Diabetes (EASD)	EASD, Amsterdam (NL)	2007
European Association for the study of Diabetes (EASD)	EASD, Rome (IT)	2008
NuGO week	NuGO, Potsdam (Ger)	2008
NuGO week	NuGO, Montecatini (IT)	2009
Wageningen Nutritional Sciences Forum	Division of Human Nutrition, Arnhem (NL)	2009
Annual meeting Society of the Study of Ingestive Behavior (SSIB)	SIBB, Portland, OR (U.S.A.)	2009
General courses and workshops		
Project & time management	VLAG, Wageningen (NL)	2007
PhD competence assessment	VLAG, Wageningen (NL)	2007
Scientific Writing	VLAG, Wageningen (NL)	2007
Information literacy	VLAG, Wageningen (NL)	2007
Good Clinical Practice	TNO, Zeist (NL)	2007
“Adviserend verkopen”	TNO, Zeist (NL)	2008
European Nutritional Leadership Program (ENLP)	ENLP, Echternach (L)	2010
Career Assessment	VLAG, Wageningen (NL)	2010
Optional courses and activities		
Preparation research proposals	WUR/TNO, Wageningen/Zeist (NL)	2007-2009
Literature group ‘Journal Club’	Division of Human Nutrition, WUR	2007-2009
Participating PhD tour U.S.A.	Division of Human Nutrition, WUR	2007
Organizing and participating PhD tour Denmark, Sweden and Finland	Division of Human Nutrition, WUR	2009
Visiting Scholar (5 months) at the Harvard School of Public Health	Harvard, Boston, MA (U.S.A.)	2010
Research presentations	TNO, Zeist	2007-2010
Research presentations	Harvard, Boston, MA (U.S.A.)	2010

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Squares are photographs of alcohol under a microscope. These photographs were taken after the alcoholic beverages have been crystallized on a slide and shot under a polarized light microscope. From top to bottom:

- Vodka
- White table wine
- Pilsner beer

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